

## ABSTRACT

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### **THE EFFECTS OF ANTIGEN VALENCY AND CpG ODN ON B CELLS**

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B cells express toll-like receptor 9 (TLR9), that recognizes microbial DNA containing unmethylated cytosyl guanosyl (CpG) sequences, induces innate immune responses and facilitates antigen-specific adaptive immunity. Studies indicate that in addition to stimulating innate immunity, TLR9 ligands can induce apoptosis in TLR9 expressing cancer cells. To understand the mechanism for TLR9-induced apoptosis, we compared the effects of CpG containing oligodeoxynucleotides (CpG ODN) on mouse primary, splenic B cells and a mouse lymphoma B cell line, CH27. CpG ODN stimulated the proliferation of primary B cells but inhibited cell proliferation and induced apoptosis in CH27 lymphoma B cells in a sequence-specific, TLR9-dependent fashion. While CpG ODN induced sustained activation of NF- $\kappa$ B and increase in c-myc protein levels in primary B cells, NF- $\kappa$ B activation was transient in the lymphoma B cells. These data suggest that the differential effects of CpG DNA on primary and lymphoma B cells occur due to differences in NF- $\kappa$ B activation. The CpG ODN-induced impaired NF- $\kappa$ B activation in the lymphoma B cells results in an imbalance between NF- $\kappa$ B and c-myc activities, inducing apoptosis in TLR9-expressing B lymphoma cells.

The B cell antigen receptor (BCR) binds to antigens in their native form. The BCR can distinguish subtle differences in antigen structure and trigger differential responses. Here, we analyzed the effects of antigen valency on the functions of the BCR using three different antigen systems – anti-BCR antibody –based antigens, phosphorylcholine (PC)-based antigens, and hen egg lysozyme (HEL)-based antigens. While both paucivalent and polyvalent antigens induced the redistribution of surface BCR into microdomains, polyvalent antigen-induced BCR microdomains persisted. Significantly, this trend was consistently observed in all three antigen systems studied. Ganglioside G<sub>M1</sub>, tyrosine-phosphorylated proteins and phosphorylated ERK colocalized with BCR microdomains, suggesting these function as surface signaling microdomains. Co-receptor, CD19 and MHC class II molecules, but not CD45 and transferrin receptor, concentrated in the BCR surface microdomains. Prolonged BCR caps were also concomitant with a reduction in BCR movement to late endosomes/lysosomes. Thus, antigen valency influences B cell responses by modulating the stability of BCR-signaling microdomains and BCR-mediated antigen transport.

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By

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## LIST OF ABBREVIATIONS

B-CLL	B-cell chronic lymphocytic leukemia
Bcl-xl	Bcl2-like 1
BCR	B cell antigen receptor
BLNK	B cell linker protein
Btk	Bruton's tyrosine kinase
CTX-B	Cholera toxin subunit B
DAG	Diacyl glycerol
ERK	Extracellular signal regulated kinase
HEL	Hen egg lysozyme
IFN- $\gamma$	Interferon- $\gamma$
Ig	Immunoglobulin
I $\kappa$ B	Inhibitor of NF- $\kappa$ B
IKK	I $\kappa$ Kinase complex
IL-6	Interleukin-6
IRAK	IL-1 receptor associated kinase 6
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun NH <sub>2</sub> terminal kinase
LAMP-1	Lysosome associated membrane glycoprotein 1
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen activated protein kinase
mDC	Myeloid dendritic cells

MHC II	Major histocompatibility complex class II
Myc	Myelocytomatosis oncogene
MyD88	Myeloid differentiation primary response gene 88
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
ODN	Oligodeoxynucleotides
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PC	Phosphorylcholine
pDC	Plasmacytoid dendritic cell
PI-3K	Phosphoinositide -3- kinase
PLC- $\gamma$ 2	Phospholipase C- $\gamma$ 2
SH2	Src-2 homology domain
SHIP	(SH2)-domain-containing inositol 5-phosphatase
SHP-1	(SH2)-domain-containing protein tyrosine phosphatase 1
TAK 1	Tumor growth factor- $\beta$ -activated protein kinase 1
TCR	T cell antigen receptor
TfR	Transferrin receptor
Tg	Transgenic mouse
TIR domain	Toll- IL-1 and IL-18 receptor domain
TLR	Toll-like receptor
TRAF6	Tumor-necrosis-factor receptor-associated factor 6
XL	Cross-linking surface BCR

## **Chapter 1: General Introduction**

The vertebrate immune system comprises the innate and adaptive immune systems. The innate immune system is an evolutionarily conserved, ancient mechanism of host defense, and is utilized by members of both the plant and animal kingdoms [1-4]. The innate immune response occurs upon recognition of highly conserved molecular structures present on invading microorganisms by the host cells. These structures, called pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs) expressed on host cells [5]. They are germline encoded, non-clonal receptors which rapidly activate effector cells. Leukocytes like macrophages, neutrophils, eosinophils, mast cells, dendritic cells, cells of the skin, and epithelia of the gut and lung express PRRs and are involved in initiating an innate immune response. The main functions of this response are phagocytosis and killing of microbes and activation of complement and pro-inflammatory pathways [6]. The adaptive immune system is a more recent evolutionary adaptation acquired only by the vertebrate phylum. The adaptive immune response is initiated by clonally distinct antigen receptors expressed by B and T lymphocytes. These antigen receptors are generated by random gene rearrangements and recognize specific features of a particular pathogen [7]. Activation of the B and T cells occurs later in the immune response and results in the generation of 'memory' cells, which allows for a stronger and quicker response on re-encountering the same microbe [5].

Upon encountering a pathogen, the innate immune response is activated rapidly and provides the initial protection, following which it facilitates the activation of the adaptive

immune response. This leads to more effective clearance of the pathogen and generates memory cells against them. Hence, vertebrates which utilize both arms of the immune response have benefited tremendously in generating optimal host defense.

### **1.1 B lymphocytes and BCR**

Among the lymphocytes involved in the adaptive immune response, the B cells are unique as they are the only cells that can differentiate into antibody-secreting cells and initiate a humoral immune response. Antibodies neutralize toxins from the invading microbe and opsonize microbes, targeting them to macrophages and complement pathways. The B cells arise from hematopoietic progenitors and differentiate in the bone marrow and migrate to the spleen as transitional B cells. In the spleen they transition from being newly emigrant T1 cells to T2 cells and then to mature B cells. The generation and maintenance of the T1 and T2 cells depends on signals from the B cell antigen receptor (BCR) and B-cell activating factor of the tumor-necrosis-factor family (BAFF) [8, 9]. Mature B cells are further classified as B1 B cells which reside in the pleural and peritoneal cavities and the conventional B2 B cells. B2 B cells include marginal zone B cells that are found in the marginal zone of the spleen and follicular B cells residing in the follicles of the lymph nodes and spleen [10]. It is hypothesized that activation of different BCR signaling components leads to the generation of different mature B cell subsets [11]. Mature, naïve, resting B cells express functional, clonally distinct antigen receptors on their cell surface.

The B cell antigen receptor (BCR) comprises of a membrane immunoglobulin (mIg) covalently associated with Ig $\alpha$ /Ig $\beta$  heterodimer. The mIg is formed by somatic recombination of a limited number of gene segments. The germline Ig light chain locus has multiple copies of three types of gene segments - variable (V), joining (J) and constant (C). The heavy chain locus has an additional fourth segment called diversity (D) segment. The variable region of the heavy chain is formed by random recombination of one each of the V, D and J segments, along with one C region. The light chain is formed by recombination of V, J and C regions [12]. This process is coordinated by the V(D)J recombinase, which includes proteins encoded by recombination-activating gene 1 and 2 (RAG1 and RAG2) and DNA-dependent protein kinase (DNA-PK). These proteins bind to specific recombination signal sequences (RSS) found adjacent to the V, D, and J segments and initiate V(D)J recombination [13]. V(D)J recombination generates heavy and light chains of varying specificities. Juxtaposing a recombined heavy with a recombined light chain increases the diversity of the functional antigen receptor. The diversity of the receptors is further enhanced by addition or deletion of non-template encoded, N-nucleotides or palindromic, P-nucleotides at the junctions between the V, D and J gene segments. The combination of these processes results in an astounding repertoire of antibodies with nearly  $10^{11}$  distinct specificities [12].

The B cell antigen receptor (BCR) comprises two components. The membrane immunoglobulin (mIg), also called surface Ig (sIg), is the ligand binding domain and the Ig $\alpha$ /Ig $\beta$  heterodimer is the signal transducing domain. The mIg is composed of two heavy chains and two light chains which have repeating immunoglobulin (Ig) domains. Each

heavy chain is linked to a light chain and to the other heavy chain by disulfide bonds. The heavy and light chains each have an N-terminal variable region and a C-terminal constant region. The variable regions of the heavy and light chains are exposed to the extracellular space and together form the antigen binding site. Mature resting B cells express mIgM and mIgD, whose heavy chains have very short cytoplasmic tails made of 3 amino acids.

The Ig $\alpha$ /Ig $\beta$  heterodimer associates non-covalently with the mIg and this association is required for mIg expression [14]. Ig $\alpha$  and Ig $\beta$  are covalently linked by disulfide bonds and each spans the membrane once. Their cytoplasmic tails are 61 and 48 amino acids respectively, containing an immunoreceptor tyrosine-based activation motif (ITAM) in each chain [15]. The ITAM is a consensus motif that is involved in signal transduction and is found in the cytoplasmic tails of many signaling molecules including CD3 and  $\zeta$  chains of the T cell antigen receptor (TCR) complex and several Fc receptors.

Phosphorylated tyrosines of the ITAMs provide binding sites for proteins with a Src-2 homology (SH2) domain and initiate signaling transduction. The individual roles of Ig $\alpha$  and Ig $\beta$  however, remain to be defined and it has been shown that they play different roles in signal transduction from the BCR [16, 17].

It was postulated that each heavy chain of the mIg associates with an Ig $\alpha$ /Ig $\beta$  heterodimer as this would allow the Ig $\alpha$ /Ig $\beta$  to neutralize the polar amino acids in each of two transmembrane domain of the mIg. Schamel and Reth, using biochemical analyses showed that every mIg associates with only one Ig $\alpha$ /Ig $\beta$  heterodimer [18]. More recently, fluorescence resonance energy transfer (FRET) studies of live cells has confirmed the 1:1

stoichiometry of mIg and Ig $\alpha$ /Ig $\beta$  heterodimer [19], although the arrangement of the mIg and the Ig $\alpha$  /Ig $\beta$  heterodimer still remains unclear.

The B cell is triggered by antigen binding to the BCR. The BCR performs two unique, yet interrelated functions. The first is to initiate and transmit signals and the second is to internalize the antigen and process it for presentation to T cells on major histocompatibility complex class II (MHC II) molecules. These two events provide the two stages of signals for B cell activation.

## **1.2 BCR signaling**

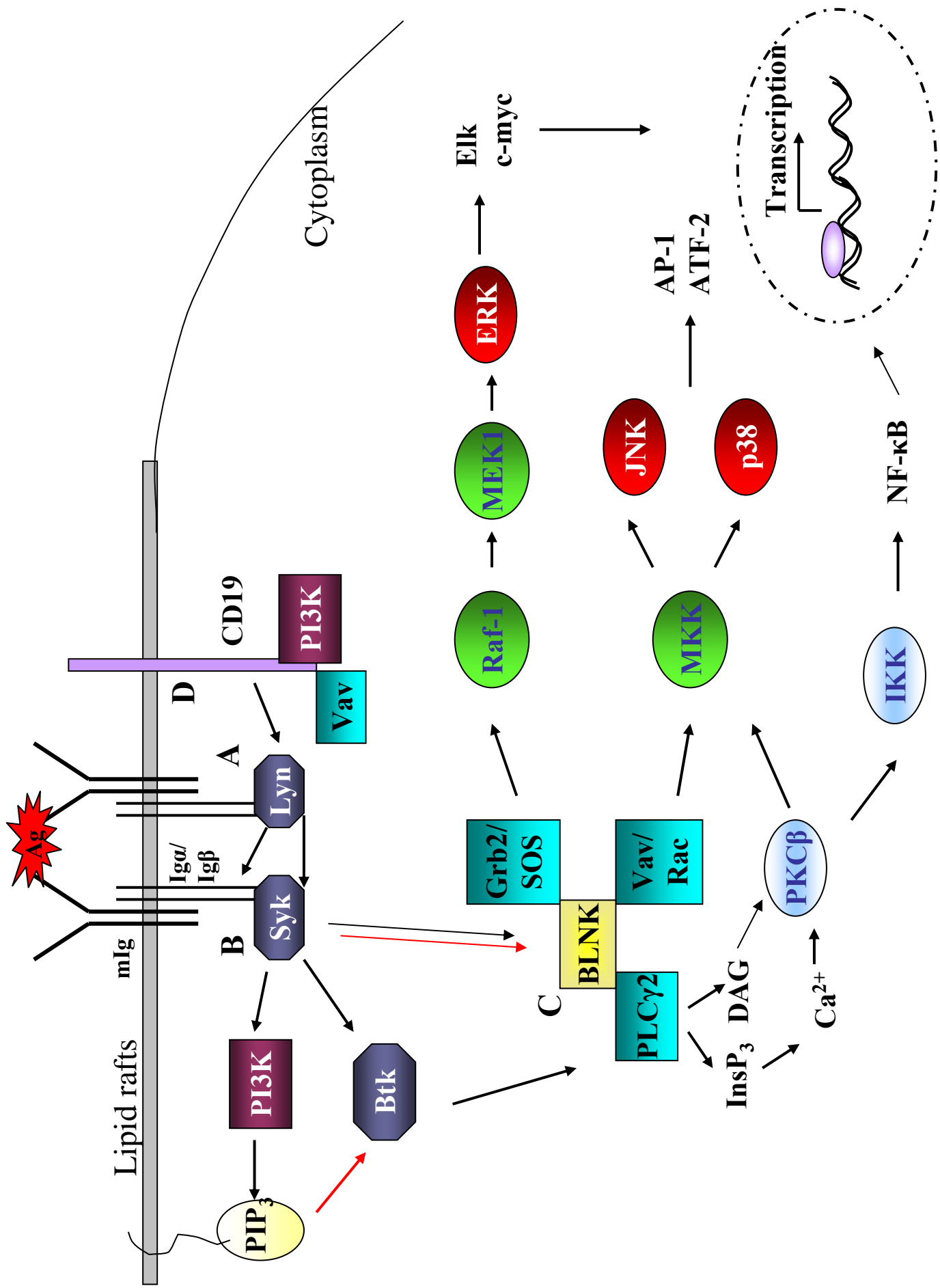
The BCR complex has no intrinsic tyrosine kinase activity. It relies on a set of protein tyrosine kinases (PTKs) that belong to the Src, Syk and Tec families for signaling.

Binding of multivalent antigens to the BCR leads to clustering of surface BCRs and association of the clustered receptors with lipid rafts (Fig. 1-1). Lipid rafts are regions of the membrane that are rich in cholesterol and glycosphingolipids and are detergent insoluble [20]. Lipid rafts provide a platform for signaling and serve to include or exclude proteins [21]. For example, in the resting, mature B cell the monomeric BCR is excluded from the rafts while the Src kinase Lyn is constitutively present in lipid rafts [20]. Oligomeric BCR clusters that move into the rafts associate almost exclusively with Lyn [22], which phosphorylates the ITAMs of Ig $\alpha$ /Ig $\beta$ . The Src kinases Fyn and Blk are also implicated in this role. Fyn and Blk are thought to be redundant to Lyn, as loss of either does not have significant impact on BCR signaling [23]. Recent evidence from fluorescence resonance energy transfer (FRET) studies suggests that receptor engagement



**Figure 1-1. BCR signaling pathway.**

Upon antigen binding the BCR translocates to lipid rafts from where it initiates the signaling cascade. Shown is a simplified schematic of the BCR signaling pathway. (A) Upon antigen binding, Src kinase, Lyn phosphorylates the ITAMs of Ig $\alpha$ /Ig $\beta$  and Syk tyrosine kinase. (B) Syk recruits and activates the adaptor protein, BLNK, PI-3Kinase and Btk. (C) BLNK provides docking sites for various proteins which amplify the antigen-induced signaling. BCR signaling culminates in the activation of transcription factors like NF- $\kappa$ B, AP-1, and ATF-2, which control gene expression. (D) In addition, accessory molecules like CD19/CD21 co-receptor complex enhance signaling by recruiting and activating Lyn, PI-3k and Vav.



leads to a conformational change of the Ig $\alpha$ /Ig $\beta$  heterodimer of the BCR from a 'closed' to an 'open' form which facilitates phosphorylation of the ITAMs by Src-family kinases [19]. The phosphorylated ITAMs amplify the signal by recruiting Syk tyrosine kinase [24]. Syk is activated by the Src kinases and by autophosphorylation [25]. Syk is a crucial player in BCR signaling as disruption of Syk impairs most downstream signaling events [26]. The adaptor protein, B cell linker (BLNK) also called SLP-65 is activated by Syk and provides docking sites for various proteins like Bruton's tyrosine kinase (Btk), phospholipase C- $\gamma$  2 (PLC- $\gamma$ 2), and adaptor proteins, Grb-2, Vav and Nck [27]. Hence BLNK is a central element in BCR signaling and BLNK-deficient mice show severe defects in BCR signaling, similar to Syk [28]. Recruitment of Btk to the proximity of Syk by BLNK makes it a target for Syk and Lyn phosphorylation and increases its activity [29]. Btk in turn phosphorylates and activates (PLC- $\gamma$ 2). In addition to BLNK, Syk also activates phosphoinositide -3- kinase (PI3K) and the Ras-Raf-ERK pathways [30, 31]. Hence, antigen binding to the BCR leads to a sequential activation of Lyn, Syk and Btk which amplifies the signal and initiates a number of downstream pathways.

CD19 that associates with complement receptor CD21, is phosphorylated by Lyn upon BCR engagement by antigen and provides binding sites for Vav, Rac and PI3K [32]. Rac along with Syk activates PI3K. Both PI3K and PLC-  $\gamma$ 2 use phosphatidyl inositol 4,5 – biphosphate (PIP<sub>2</sub>) as their substrate. PI3K phosphorylates PIP<sub>2</sub> to phosphatidyl inositol 1,4,5 – trisphosphate (PIP<sub>3</sub>) which recruits molecules with pleckstrin homology (PH) domains, like Btk, to the BCR signalosome. PI3K also activates Akt, a serine-threonine kinase, which promotes proliferation by inhibiting BAD, a pro-apoptotic protein. PLC-  $\gamma$ 2

hydrolyzes PIP<sub>2</sub> to inositol trisphosphate (InsP<sub>3</sub>) and diacyl glycerol (DAG), which releases intracellular calcium and activates protein kinase C  $\beta$  (PKC- $\beta$ ). PKC- $\beta$  activates nuclear factor- $\kappa$ B (NF- $\kappa$ B), which upregulates the anti-apoptotic protein Bcl-xl and cyclin D2 [33]. PKC- $\beta$  and Rac activate mitogen activated protein kinases (MAPKs) – p38, c-Jun NH<sub>2</sub> terminal kinase (JNK), and extracellular signal regulated kinase (ERK) [34]. The MAP kinases activate specific transcription factors, like c-jun, c-fos, Elk and c-Myc which along with NF- $\kappa$ B and NFAT impact the cellular response to antigen engagement [35].

BCR signaling is influenced by receptor-associated accessory molecules. CD19/CD21 complex enhances signaling by the BCR by recruiting and activating Lyn, PI-3k and Vav [32], and is essential for B cell response to thymus-dependent protein antigens [36]. CD22, the paired immunoglobulin-like receptor B (PIRB) and Fc $\gamma$ RIIB are negative regulators of BCR signaling. These molecules have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. They recruit phosphatases Src-homology-2 (SH2)-domain-containing inositol 5-phosphatase (SHIP) and (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1) which serve to down regulate and inhibit BCR signaling [37, 38]. Interestingly these negative regulators, like CD22 and SHP1, are excluded from the lipid rafts [20].

A model of BCR signaling has started to emerge where the BCR is thought to actively assemble a signaling complex or ‘signalosome’ in the lipid raft where it is protected from negative regulators like phosphatases. Thus the B cell receptor uses a number of effector

enzymes, adaptor proteins and the lateral heterogeneities of the plasma membrane to regulate the precision of its signal transduction and influence cell fate decisions.

### **1.3 Cellular responses of B cells**

Humoral immune responses are initiated by antigen binding to the B cell antigen receptor.

The developmental stage of the B cells, the nature of the antigenic stimulus and the availability of T cell-dependent and independent stimuli control the cellular response of B cells. Splenic marginal zone (MZ) B cells and peritoneal B1 B cells are the first responders which sense T-cell independent antigens like bacterial polysaccharide.

Follicular B cells respond to protein antigens and require the help of antigen-specific CD4<sup>+</sup> T cells. T-dependent responses lead to the formation of memory B cells, a hallmark of adaptive immunity. In both cases activation of B lymphocytes is a multi-step process where many external signals are integrated to result in a cellular response.

#### ***1.3.1 Thymus-Independent (TI) antigens***

There are two types of thymus-independent (TI) antigens. Type 1 TI antigens like bacterial lipopolysaccharide (LPS) and peptidoglycan are polyclonal B cell activators which are Toll-like receptor (TLR) ligands and induce non-antigen specific B cell responses. Type 2 TI (TI-2) antigens induce antigen-specific B cell activation [39]. TI-2 antigens are characterized by large molecular weight and multiple repeating antigenic epitopes that mediate extensive cross-linking of BCR. Antigens like polysaccharides and lipids present on the surface of capsulated bacteria, the cell wall of non-encapsulated bacteria and viral capsids are TI-2 antigens [40]. TI-2 antigens, like phosphorylcholine

expressed on bacterial cell walls effectively activate MZ and B1 B cells and induce proliferation, Ig secretion and plasma cell differentiation [41]. It has been suggested that TI-2 antigen induced Ig secretion requires a second signal from T-independent sources. B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) expressed on macrophages, monocytes and dendritic cells have been implicated in this role. They bind to transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA) on B cells and modulate survival and activation [42, 43], and are essential for B cell response to TI antigens [42]. TACI-deficient mice do not respond to TI-2 antigens [44]. Microbial products like LPS, bacterial lipoproteins and bacterial DNA containing the unmethylated CpG dinucleotide motif activate the Toll-like receptor (TLR) pathway and synergize with TI-2 antigens to enhance B cell proliferation and Ig secretion [45-47], potentially providing a second signal for TI-2 antigen-triggered B cell activation. Furthermore, TI-2 antigens can activate the complement pathway, which lowers the threshold of antigen needed for B cell proliferation [48] and BCR signaling threshold through CD19/CD21 co-receptor. B cells exposed to TI antigens undergo proliferation, class switching and can form germinal centers. This is exemplified by the formation of germinal centers (GCs) when mice lacking CD3<sup>+</sup> T cells are immunized with NP-Ficoll, a prototypical TI-2 antigen [49]. However, these GCs are short-lived and the cells do not undergo somatic hypermutation to differentiate into memory B cells. Thus TI-2 antigens activate the host to rapidly produce antibodies with help from non-T cell sources, which prevents the multiplication and spreading of invading microbes in the early stage of infection.

### ***1.3.2 Thymus-dependent (TD) antigens***

B cells typically detect soluble antigens but they can also interact with native antigens that are presented by antigen presenting cells like follicular dendritic cells. B cells that recognize membrane-bound antigen spread over these antigen presenting cells and then contract to accumulate and extract the antigen [50]. Naïve B cells which form an immune synapse with antigen presenting dendritic cells rearrange their BCRs to form a central ring and excluding negative co-receptors like CD45 and CD22 [51]. Adhesion molecules like ICAM-1 and VCAM-1 on the antigen-tethered cells interact with LFA-1 and VLA-4 on the B cells, creating a docking site for the B cells on the target cells [52, 53]. This could reduce the threshold of B cell activation since the formation of such synapses could be more potent in activating the B cells.

T-dependent (TD) responses require the recognition of antigen by cognate B and T lymphocytes. Lanzavecchia showed that this occurs by the recognition of native antigen by BCR and processed antigen by TCR in the context of MHC class II [54]. Real time imaging analysis of mice injected with hen egg lysozyme (HEL) antigen shows that the antigen experienced B cells migrate to the B cell-T cell boundary of the lymph nodes [55], where antigen activated T and B cells sample each other and establish interaction with cells of cognate antigen specificity. The B-T conjugate lasts for about 20-40 min [55]. An extensive and active rearrangement of the surface molecules takes place in both cells forming the immune synapse. The TCR which recognizes cognate MHC class II and kinases like ZAP70 and Lck, migrate to the centre of a ring surrounded by molecules involved in cell adhesion like LFA-1 and talin [56, 57]. This structure is called a supra

molecular activation cluster (SMAC) [58]. The TCR forms microclusters within 30 seconds of stimulation and initiates signaling, before merging together as the central SMAC or cSMAC [59]. Upon formation of the cSMAC the TCRs stop signaling and are sorted for degradation [60].

Importantly upon B-T conjugate formation, cytokines secreted by T cells and signaling triggered by the interaction of co-stimulatory molecules on both cells activate B cells to undergo clonal expansion and differentiation into antibody-secreting cells. CD40 which is constitutively expressed on B cells binds CD154 or CD40 ligand (CD40L) on activated T cells and is essential for class switching and GC formation [61, 62]. Class switching refers to the process of isotype switching of the heavy chain from  $\mu$  and  $\delta$  to  $\gamma$ ,  $\alpha$  and  $\epsilon$ . This occurs by a mechanism called class switch recombination (CSR). During this process the rearranged VDJ gene segment of the heavy chain recombines with downstream  $\gamma$ ,  $\alpha$  or  $\epsilon$  C regions leading to production of IgG, IgA or IgE antibodies. Inducible costimulator (ICOS) present on T cells binds its ligand (ICOSL) on B cells and acts upstream of CD40 [63]. ICOS-deficient mice do not undergo CSR or GC formation [64]. ICOS belongs to the B7-CD28 family. Members of this family, including B7.1 and B7.2 on B cells and CD28 on T cells, are upregulated sequentially. While they are crucial in the immunologic synapses between DCs and T cells, their importance in the immunological synapses between B and T cells is unclear [65]. OX40-OX40L and CD27-CD70 interactions have also been implicated in the co-stimulation provided by B-T cell interaction [54]. Activated CD4<sup>+</sup> T cells secrete cytokines that along with co-stimulatory molecules stimulate B cell proliferation and differentiation. Cytokines are



soluble proteins that mediate effector functions of cells. Activated CD4<sup>+</sup> T cells secrete IL-2, IL-4 and IL-5 to promote B cell proliferation and isotype switching. IL-6 is important for growth of IgM-secreting plasma B cells. CD40 ligand and cytokines secreted by CD4<sup>+</sup>T cells activate B cell proliferation and Ig secretion.

Upon stimulation by cognate T cells, the B cells undergo clonal expansion and differentiate into short-lived plasma cells or enter the lymphoid follicle to form germinal centers (GCs). The short-lived plasma cells secrete germ-line encoded antigen-specific antibodies [66]. The B cells that enter the lymphoid follicle form a germinal center where they rapidly proliferate and diversify their BCR through somatic hypermutation (SHM) [67]. During SHM, the VDJ segments of Ig heavy chain and VJ segments of Ig light chain typically undergo base substitution or small deletions. The BCRs accumulate one mutation with each cell division. B cells expressing BCRs with higher affinity are positively selected by cognate T cells in the germinal center [68]. Hence this process is also called affinity maturation. Upon exiting the GC they differentiate to form long-lived memory B cells or long-lived plasma cells. The long-lived plasma cells home to the bone marrow and secrete high affinity antibodies, which serves to maintain antibody levels for the life of the animal. The affinity-matured memory B cells are non-secreting cells that circulate or home to the marginal zone of the spleen and rapidly proliferate and differentiate to plasma cells upon antigen reencounter [69]. Thus B cells reacting to T-dependent antigens give rise to antibodies and memory cells which are considered cardinal features of a successful adaptive immune response.

Naïve B lymphocytes integrate signals from two cascades for successful expansion, class switching and Ig secretion. Signal 1 comes from binding of native antigens to the BCR. Signal 2 arises from the recognition of processed antigen on MHC II by the TCR, T cell-secreted cytokines and interaction of B cells and T cells through co-stimulatory molecules like CD40-CD40L [70]. The ‘two signal’ model proposed by Bretscher and Cohn still stands as the dogma of naïve B cell activation [71].

#### **1.4 Role of antigen properties in B cell responses**

The B cell antigen receptor recognizes antigens in their native forms with varying affinity, valency, chemical component and stereochemical structure. Antigen binding to BCR initiates cellular events that can lead to anergy, apoptosis, proliferation, differentiation, or memory B cell generation. The potential of the B cell to induce qualitatively disparate cellular responses is a result of the ability of the receptor to distinguish subtle differences in the nature of the ligand that interacts with it. The cellular response of B cells is influenced by the properties of antigens, differentiation stage of B cells, availability of T cell help, and environment of B cells. The relationship between antigen affinity and antigen-triggered responses has been extensively studied. From transgenic mice that expressed BCR specific for hydroxyl-nitro phenyl acetate (NP), Shih et al. generated mice that express BCR with high and low antigen binding affinity. High- and low-affinity B cells responded similarly to T-dependent antigens, although only high-affinity B cells accumulated in the germinal center [72] and proceeded to form long-lived plasma cells [73]. High-affinity B cells generated a two-fold higher response to T cell-independent antigens than low-affinity B cells [74]. Increasing antigen-BCR affinity has

been shown to enhance B cell antigen presenting ability, T cell-independent B cell proliferation, antibody secretion, and interleukin 2 and interferon- $\gamma$  secretion *in vitro* [75, 76].

Antigen valency also influences B cell responses. When antigen valency is increased by conjugating BCR-specific antibodies to dextran, Ficoll, Sepharose, or polyacrylamide beads, the amount of antigen required for induction of B cell proliferation is reduced [77, 78]. Increases in antigen valency synergized with CD40 ligand to induce Ig synthesis and isotype switching in splenic B cells [79]. Conversely, increasing antigen valency using biotinylated anti-BCR antibodies and avidin or by coating anti-BCR antibodies to plastic or erythrocyte surface induced abortive activation of B cells and apoptosis [80-82].

Recently, Kim et al., reported that oligomeric antigens are more effective than monomeric antigens in inducing rearrangement of MHC class II-containing compartments and enhance antigen presentation by B cells [83]. Antigen valency and affinity also regulate the selection of activated B cells to differentiate into short-lived extrafollicular antibody-secreting plasma cells or enter the germinal center. Decreasing antigen valency or affinity reduces the number of B cells forming extrafollicular plasma cells [84]. Our published study demonstrated that increasing antigen valency increases the stability of BCR surface signaling microdomains, enhances the level and duration of antigen-triggered protein tyrosine phosphorylation, and decreases BCR internalization and transport [85]. Thus, the property of antigens is an important factor that regulates B cell responses.

In this dissertation, I have determined the effects of varying antigen valences on BCR functions. Based on published work and our previous study [85], we hypothesized that increasing binding valency of antigen would lead to the formation of stable BCR signaling microdomains that trigger strong, prolonged signaling by differentially recruiting signaling molecules. Using three different model antigen systems, I extended our previous observations by comparing the composition of BCR surface signaling microdomains and kinetics of recruitment of key signaling molecules to the BCR surface microdomains induced by antigens of varying valences.

### **1.5 Role of Toll-like receptors (TLR) in B cell responses**

There is growing evidence of the importance of Toll-like receptors (TLRs) in the activation and physiology of B cells. Recent studies suggest that TLR signaling can act as a third signal and influence activation of naïve B cells [86, 87]. Using naïve human B cells, Ruprecht and Lanzavecchia showed that while BCR triggering and T cell help induced B cell proliferation, the cells died after 4 cycles of division. CpG ODN synergized with BCR stimulation and T cell help to induce sustained B cell proliferation [86]. Antigen-specific antibody responses in MyD88 knockout mice were observed to be reduced. Adoptively transferring MyD88 KO B cells into a mouse that lacks mature B cells, induced lower levels of IgM and IgG<sub>1</sub> antibodies, while IgE levels were normal [87]. Further both human and mouse B cells required TLR stimulation for formation of germinal centers and differentiation of B cells to antibody-secreting plasma cells [86, 87]. TLRs can stimulate B cells directly or through cytokines secreted by activated dendritic cells and T cells. Although all TLR agonists can function as the third signal, agonists of

TLR2 and TLR9 activated human B cell proliferation more extensively than agonists of TLR3 or TLR5 [86]. Remarkably, in sharp contrast to murine B cells which can be activated by TLR agonists to undergo polyclonal proliferation, human B cells respond to TLR signaling only after receiving initial BCR stimulation. This coupling of BCR and TLR signaling helps to maintain specificity of the immune response by focusing innate immune signals on antigen stimulated B cells.

Using MRL-lpr mice, a well studied model of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), Leadbetter et al. [88] have implicated co-ligation of TLR9 and BCR in the pathogenesis of these autoimmune diseases. Immune complexes of self-DNA and antibody activate self-reactive B cells by co-ligating TLR9 and BCR, leading to production of autoantibodies [88, 89]. The BCR seems to act as a conduit delivering the self DNA to the endosomal compartments where TLR9 is present. Upon extensive cross-linking of the BCR even DNA that lack the optimal CpG motif and are methylated are recognized by TLR9 and lead to antibody production [45]. Taken together these results suggest that extensive cross-linking of the BCR may broaden the specificity of TLR9 and trigger autoantibody secretion upon encountering non-immunostimulatory self DNA. This indicates that antigens signaling through the BCR and autoantigens through TLRs may contribute to the loss of peripheral B cell tolerance and lead to the induction of autoimmune diseases.

## **1.6 Toll-like Receptors**

Pathogen recognition receptors (PRRs) are involved in the innate immune response. Toll-like receptors (TLRs) are the best characterized of the PRRs. The *Toll* gene in *Drosophila* was cloned as a gene that participated in dorso-ventral axis formation in the fly embryo [90]. Lemaitre et al. demonstrated that loss-of-function mutation in the *Toll* gene led to increased susceptibility to fungal infections in flies [4], suggesting a possible role for *Toll* in the immune response. A human homologue of *Toll*, Toll-like receptor (TLR) 4, was first cloned and characterized by Medzhitov et al. [3]. To date 10 TLRs have been identified in humans and 13 in mice. They differ in ligand specificities and expression profiles [91]. While they utilize a common signaling pathway that leads to the secretion of pro-inflammatory cytokines, TLRs can also activate alternate signaling pathways that induce pathogen-specific responses [92].

### **1.6.1 TLR ligands**

TLRs recognize specific microbial components and initiate an immune response to bacteria, fungi, protozoa and viruses. TLR2 functions as heterodimer with TLR1 or TLR6 and binds diacyl lipoproteins and triacyl lipoproteins respectively [93]. TLR4 is the essential receptor for lipopolysaccharide (LPS) present on Gram-negative bacteria [94]. TLR4 has also been shown to bind endogenous ligands like heat shock proteins, fibronectins, hyaluronic acid and fibrinogen when they are present in high concentrations [93]. TLR5 is the receptor for flagellin, a component of bacterial flagella [95]. The above mentioned TLRs have all been shown to be expressed on the cell surface by staining with specific antibodies [93]. Nucleic acids from viruses and bacteria are recognized by a host

of TLRs that are expressed intracellularly in endosomes. TLR3 binds to double stranded viral RNA (dsRNA) [96], while viral ssRNA is recognized by TLR7 and human TLR8 [97]. TLR9 binds hypomethylated DNA sequences with CG motifs (also called CpG DNA) that are present in much higher frequency in prokaryotes than in eukaryotes [98]. A profilin-like molecule from *Toxoplasma gondii* is the known ligand for mouse TLR11, which also mediates responses to uropathogenic bacteria [99, 100].

### **1.6.2 Structure of TLRs**

The TLRs are type I integral membrane glycoproteins with an N-terminal ectodomain (ECD), a single transmembrane domain and a cytoplasmic tail containing the Toll- IL-1 and IL-18 receptor (TIR) domain [101].

Individual TLR ectodomains (ECDs) can recognize multiple ligands from both endogenous and exogenous sources [101, 102]. Initial analysis of the TLR sequences indicated the presence of multiple copies of conserved leucine-rich repeats or LRRs. The LRR is a 24 amino acid sequence with characteristically placed hydrophobic residues. The LRR in the Toll-like receptors has the following consensus sequence: X L X X L X L X X N X  $\phi$  X X  $\phi$  X X X X F X X L X where X is any amino acid, L is leucine, N is asparagine, F is phenylalanine and  $\phi$  is any hydrophobic residue [103]. Molecular models based on known structures of proteins like ribonuclease inhibitor, CD42b, SDS22, a yeast protein that contain LRR proposed that the 19-25 LRRs in the TLR ectodomain would form a horse-shoe shaped solenoid, with a concave surface consisting of  $\beta$ -strands and a convex surface that lacks defined secondary structure. Recent evidence from the crystal

structure of TLR3 supports the proposed models [104, 105]. The crystal structure also reveals that the N- and C- terminals of the ECDs are capped by cysteine-rich residues that protect and stabilize the hydrophobic core. Individual TLRs are distinguished by modifications of specific LRR [103]. For example, TLRs 7, 8 and 9 are characterized by insertions at LRRs 2, 5, 8 and 11 that differ from the consensus sequence. It has been suggested that these structural variations which occur on the concave part of the solenoid provide different TLRs the specificity to bind different ligands [103, 105]. In theory, a combination of insertions could give the  $\beta$  surface of the TLR ECD 10 times greater binding area than the surfaces of antibodies [103]. Another hypothesis is that the binding surface for TLR3, lies on the convex side, in the V-shaped valley formed upon dimerization of the TLRs [104]. Since TLRs have been shown to cluster hetero- and homo-typically even in the absence of ligands [106, 107], this raises the intriguing possibility that the inactive TLRs start as dimers that rearrange upon stimulation, to recognize specific ligands [104, 108].

The cytoplasmic TIR domain is made of 200 amino acids. There are three conserved boxes that are crucial for signaling from the TLR. Deletion and substitution of specific amino acid residues in these boxes abrogates signaling activity of the receptor [109, 110]. Structure analysis of the TIR domains of TLR1 and 2 has been performed. The TIR domains contain 5 parallel  $\beta$ -strands flanked by 5  $\alpha$ -helices on both sides. The  $\beta$ -strands are connected to the  $\alpha$ -helices by loops [111]. The TIR domains of proteins studied so far, show 20-30% sequence conservation. There are large insertions or deletions in several loop regions, which may account for the diversity in signaling initiated from the different



TLRs. Structural studies suggest that conserved surfaces of the TIR domain may mediate oligomerization of the TLRs and facilitate interactions between the TLRs and downstream adaptor molecules, like myeloid differentiation primary response gene 88 (MyD88), that contain TIR domains [111]. For example, the Lps<sup>d</sup> mutation which alters the sequence in one of the loops of TLR4 abolishes LPS-induced signaling, by interfering with a point of contact between the receptor and downstream adaptors [94].

### **1.7 Toll-like receptor 9 and CpG DNA**

For more than a century, bacterial extracts like bacillus Calmette Guerin (BCG), have been used to treat cancer and allergies and more recently as vaccine adjuvants [112]. It was discovered that the active component of the bacterial extract that induced tumor regression was DNA [113], and later the consensus functional motif for this DNA was identified as XCGY. The C is hypomethylated (hence CpG) and X is any base other than C, and Y is any base but G [114]. In the vertebrate genome CG dinucleotides occur only at one quarter of the expected frequency due to active CG suppression. While bacterial and some viral DNA contain largely unmethylated cytosines, almost 70% of cytosines in vertebrate DNA are methylated [115]. Recognition of this structural difference between DNA of microbes and vertebrates is postulated to be an evolutionary mechanism of effective defense against pathogens. Recognition of CpG motifs triggers an immune response similar to those activated by LPS and other common microbial products [114].

### ***1.7.1 Toll-like receptor 9***

Toll-like receptor 9 (TLR9) knockout (KO) mice do not respond to CpG DNA, suggesting that TLR9 is the receptor for CpG DNA [98]. Human embryonic kidney 293 (HEK293) cells transfected with TLR9 become responsive to CpG DNA [116]. Ligand binding studies in these cells have shown that ssCpG DNA with phosphodiester backbone binds directly to TLR9 [117]. This binding is sequence-specific at acidic pH conditions, like those of the endosomes [117, 118]. DNA containing a phosphorothioate backbone can also bind TLR9 sequence non-specifically [119], although only CpG DNA activates TLR9 [117]. This is significant since most studies of CpG DNA use DNA with phosphorothioate backbone modification as this makes it resistant to nucleases and stimulates cells better. TLR9 is primarily expressed in B cells and plasmacytoid dendritic cells (pDC) in humans [112, 120]. Neutrophils [121] and epithelial cells of the lung [122] have also been reported to express TLR9. While murine and human NK cells [123] and T cells [124] secrete interferon- $\gamma$  (IFN- $\gamma$ ) upon treatment with CpG DNA, this effect is indirect and depends on the presence of adherent cells or their secreted molecules [125]. However, the expression pattern of TLR9 in mice is different, and it has been reported to be expressed on macrophages and myeloid dendritic cells (mDCs) in addition to B cells and pDCs [120]. This divergence in expression profile of TLR9 makes it difficult to extrapolate the effects of CpG DNA from murine models to the human system although human clinical trials initiated based on preliminary research in mouse models show promise for the use of CpG DNA as a therapeutic [126, 127].

### ***1.7.2 CpG DNA***

The optimal CpG motif for activating TLR9 expressing mouse cells is GACGTT [128] and for human cells it is GTCGTT [129]. Empirical studies of structure-activity relationships have shown that the immune response to CpG DNA is altered by the number and spacing of CpG motifs, modification of the backbone and presence of poly G motifs. Based on these studies three classes of CpG ODN have been described [130]. Class A ODNs typically contain a central phosphodiester backbone with phosphorothioate ends and poly G motifs. They induce IFN- $\alpha$  secretion from pDC, but are poor activators of pDC maturation and B cells [112]. B cells are stimulated by class B CpG ODN, which have fully phosphorothioate backbones. These also promote maturation of pDCs but do not induce IFN- $\alpha$  secretion [112]. The C-class CpG ODN have properties that are considered intermediate of A and B classes, inducing IFN- $\alpha$  secretion from pDC and activating B cells [130, 131]. Oligonucleotides that suppress TLR9 signaling have also been reported. The S-class ODN seem to block CpG ODN-induced NF- $\kappa$ B and AP-1 activation [132, 133] and IL-12 and IFN- $\gamma$  secretion [134], although the precise mechanism of action is not completely understood. In this dissertation, we have used a B-class CpG ODN (1826 or CpG DNA) and its control (1826GC or GpC DNA). This sequence has been shown to be an optimal activator of mouse B cells [130].

## **1.8 TLR9 signaling**

### ***1.8.1 Cellular internalization of CpG ODN***

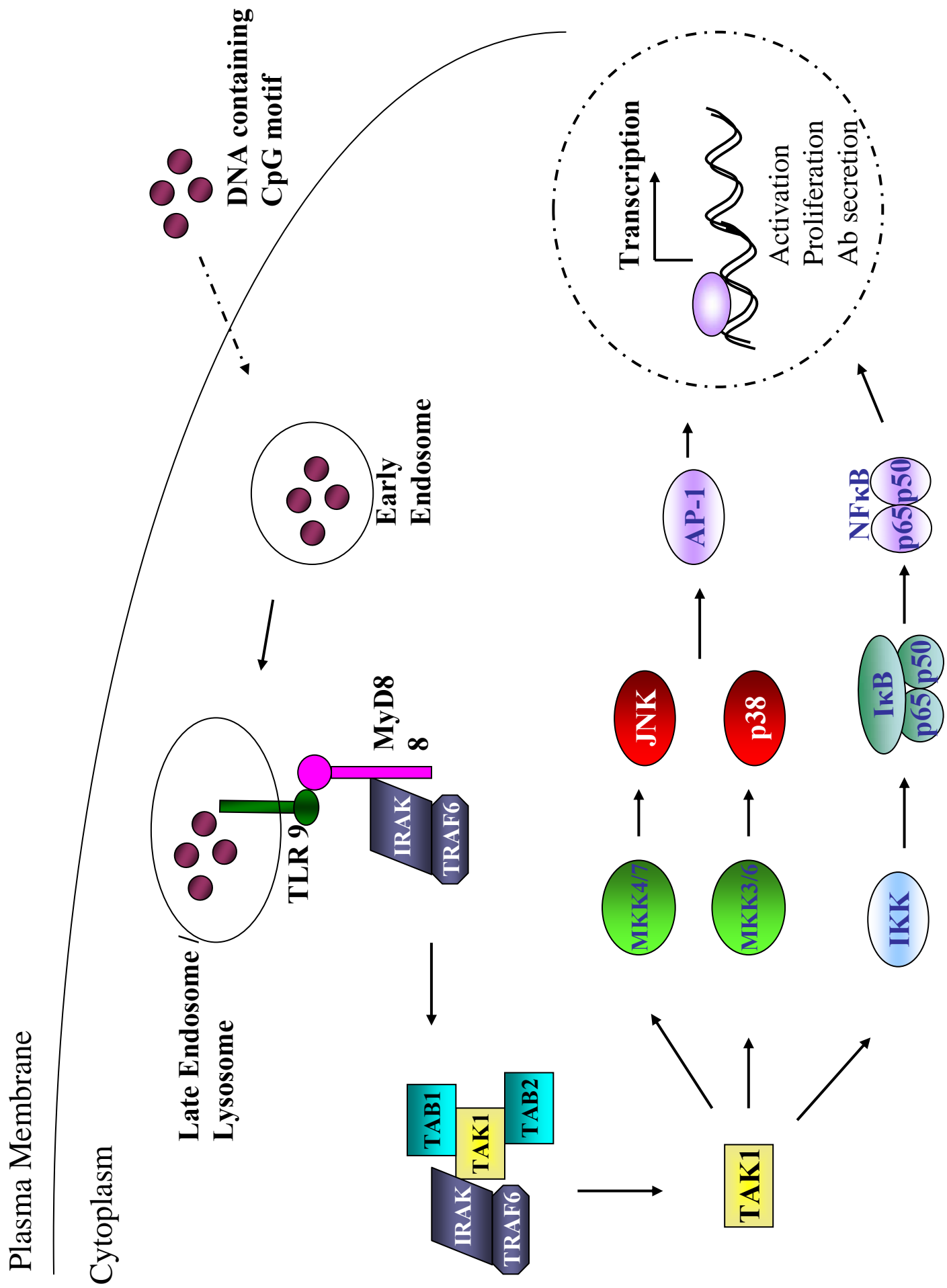
Oligonucleotides are large polyanions that cannot diffuse freely across the cell membrane. ODNs are taken up by cells through pinocytosis and possibly through receptor-mediated endocytosis [135]. While no single receptor has been implicated in this role, it is postulated that cell surface DNA binding proteins that bind ODNs in a sequence independent manner [114] might help transport them across the plasma membrane. CpG DNA briefly associates with the cell surface and rapidly enters into endocytic vesicles, presumably endosomes, near the plasma membrane in a Rab5-dependent process [136, 137]. This is a temperature and energy-dependent, but sequence independent process. Treatment of macrophages and B cells with Bafilomycin A and chloroquine abrogates CpG DNA-induced cellular activation [138, 139]. Bafilomycin and chloroquine are inhibitors of endosomal H<sup>+</sup> ion pumps that increase endosomal pH and block endosomal maturation. These data indicate that the endosome is a site where CpG DNA initiates signaling (Fig. 1.2). In pDCs, CpG DNA is transported from cell surface by endosomes to tubular lysosomal structures in the perinuclear region [119]. In macrophages, the DNA positive vesicles co-localize with lysosome-associated membrane glycoprotein-1 (LAMP-1), a marker for late endosomes and lysosomes [136]. These data suggest that CpG enters the cell into early endosomes and rapidly moves into the lysosomal compartment.

Endogenous and transfected TLR9 is intracellular, expressed and located in the endoplasmic reticulum (ER) in pDCs and macrophages [119, 140]. Flow cytometric

**Figure 1-2. The TLR9 signaling pathway in B cells**

Oligonucleotides containing the CpG motif are internalized by B cells into endosomes.

TLR9 encounters the ODN in late endosomal/lysosomal compartments. This initiates signaling from TLR9 via MyD88-IRAK-TRAF6-TAK1. TAK1 leads to the activation of I $\kappa$ B kinase (IKK) and MAP kinases, JNK and p38. The kinases activate transcription factors like, NF- $\kappa$ B and AP-1 which regulate gene expression.



analyses of various B cell lines shows very little or no TLR9 expression on the cell surface [140]. Results from microscopy studies of macrophages and pDCs, show that upon stimulation with CpG DNA, both MyD88 and TLR9 are actively recruited to the CpG containing vesicles [119]. In pDCs, TLR9 is redistributed from the ER to CpG-containing lysosomes where it initiates signal cascades [119, 141]. The mechanism of how TLR9 redistributes from the ER to the signaling compartment remains unclear. Proteins that are targeted to the plasma membrane from the ER, normally travel through the Golgi apparatus and the secretory pathway. The carbohydrates on these proteins acquire resistance to cleavage by Endoglycosidase H (Endo H), as they are modified by the Golgi enzymes. TLR9 that reaches the CpG-containing endosomes, remains sensitive to Endo H, raising the possibility that it does not pass through the Golgi [119]. Other possible pathways include a direct fusion of the ER to the endosome or to the plasma membrane.

pDCs internalize CpG DNA into endosomes and transport them into tubular lysosomes as early as 10 min of stimulation [119]. Dendritic cells are unrivalled in their efficiency as antigen presenting cells. They are equipped with unique antigen presenting pathways, which allows them to effectively and rapidly prime naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells [120]. Naïve, resting B cells on the other hand, are not very efficient APCs. It is likely that B cells do not internalize and transport DNA in a similar way as the pDCs. While internalization of CpG DNA [114] and endosomal acidification [139] have been reported to be essential for CpG-induced stimulation of B cells, the kinetics and transport pathway

of CpG ODN in murine B cells remain to be studied. As part of this dissertation, I report the visualization and kinetics of the internalization of CpG ODN in mouse B cells.

### ***1.8.2 Proximal TLR9 signaling events***

Despite the diversity of ligands, TLRs activate similar signaling pathways. Following internalization of CpG into endosomes, MyD88 and TLR9 are actively recruited to these vesicles [136]. MyD88 has emerged as a key adaptor shared by many TLRs. While some TLRs use MyD88-independent pathways [142], TLR9 signaling is MyD88-dependent and MyD88 KO mice do not respond to CpG stimulus [143]. The TLR-MyD88-IRAK4-IRAK1-TRAF6 forms the classical TLR signaling complex and is functional in signaling cascades of all TLRs except TLR3 [92]. TLR activation leads to sequential phosphorylation of IRAK4 and IRAK1 by MyD88. IRAK1 in turn recruits and phosphorylates tumor-necrosis-factor receptor-associated factor 6 (TRAF6). MyD88 KO mice and TRAF6-deficient cells do not respond to CpG DNA stimulus, indicating their essential role in TLR9 signaling pathway [143]. TRAF6 activates tumor growth factor- $\beta$ -activated protein kinase 1 (TAK1), which forms a complex with TAB1, TAB2 and TAB3 [144]. Although a direct substrate of TAK1 remains to be identified, it activates I $\kappa$ B kinase complex (IKK) and NF- $\kappa$ B. TAK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and activates MAP kinases JNK, p38 and ERK [145].



### ***1.8.3 Activation of Nuclear Factor- $\kappa$ B (NF- $\kappa$ B)***

CpG DNA-triggered TLR9 signaling pathway activates NF- $\kappa$ B in mature mouse splenic B cells and human B cells [129, 146]. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) was identified 20 years ago as a nuclear factor that bound to the enhancer regions of the  $\kappa$  light chain in B cells [147, 148]. Mammalian NF- $\kappa$ B family of transcription factors refers to five DNA binding members, p65 (Rel-A), c-Rel, RELB, p50 (a processing product of p105, also called NF- $\kappa$ B1) and p52 (a processing product of p100, also called NF- $\kappa$ B2). These proteins form homo- or hetero-dimers of functional NF- $\kappa$ B [149]. The p65/p50 hetero-dimer is considered the prototypical NF- $\kappa$ B complex. The NF- $\kappa$ B family is characterized by the presence of the rel homology domain (RHD); which contains motifs that promote dimerization, DNA binding and nuclear localization. In addition to the RHD, p65, c-Rel and RelB also have a transactivation domain [150].

In unstimulated cells, the NF- $\kappa$ B dimers are rendered inactive by association with one of the inhibitors of NF- $\kappa$ B (I $\kappa$ B) in the cytoplasm. The I $\kappa$ B family comprises I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$  and I $\kappa$ B $\epsilon$ . The I $\kappa$ Bs interact with the NF- $\kappa$ B family through ankyrin repeats, block the nuclear localization signal of NF- $\kappa$ B and hold the NF- $\kappa$ B complex in the cytoplasm. Upon stimulation of the cell with CpG ODN, I $\kappa$ B is phosphorylated by I $\kappa$ B Kinase (IKK) complex, which leads to polyubiquitination and degradation in the proteasome. The free NF- $\kappa$ B dimers translocate to the nucleus and bind specific NF- $\kappa$ B binding motifs in promoter regions [150]. NF- $\kappa$ B is a key factor that regulates the transcription of genes related to host defense. In addition, NF- $\kappa$ B also activates the transcription of I $\kappa$ B $\alpha$ . Free

I $\kappa$ B $\alpha$  binds NF- $\kappa$ B in the nucleus and promotes its export to the cytoplasm via an amino-terminal nuclear export sequence [151]. This helps restore NF- $\kappa$ B to its resting state.

NF- $\kappa$ B plays an important role in innate and adaptive immune responses by controlling the transcription of a number of genes that are essential in host defense. NF- $\kappa$ B is activated by more than 200 different physiological stimuli and stimulates the expression of hundreds of target genes, including cytokines, immune receptors, regulators of cell cycle and apoptosis, and other transcription factors [152]. It also plays an important role in the generation and maintenance of mature B cells. Rel-A/p65-deficient B cells have greatly diminished numbers of B cells and are sensitive to TNF-mediated apoptosis [153]. Furthermore, NF- $\kappa$ B is constitutively activated in a number of human B cell lymphomas [154, 155] and is possibly required for maintaining the tumorigenicity of these lymphomas [156, 157]. The role of NF- $\kappa$ B in inducing growth and antagonizing cell death is well established. NF- $\kappa$ B induces the cell cycle progression by activating genes like cyclin D1 [158], c-myc [159] and c-myb [160]. NF- $\kappa$ B prolongs cell survival through upregulation of anti-apoptotic genes like Bcl-xl, c-IAP-1, c-IAP-2 and XIAP [161, 162]. Hence, NF- $\kappa$ B is generally considered an anti-apoptotic transcription factor; although it can also mediate cell death under certain conditions. T cells exposed to the oncoprotein, Tax, from human T-cell leukemia virus-I (HTLV-I) underwent apoptosis by upregulating TNF-related apoptosis inducing ligand (TRAIL) in an NF- $\kappa$ B -dependent fashion [163]. NF- $\kappa$ B has also been reported to upregulate Fas [164] and Fas ligand [165], while suppressing pro-survival genes like Bcl-xl and c-IAP [166, 167] in various cell lines. Thus NF- $\kappa$ B is a crucial mediator of cell survival and death in many types of cells,

including B lymphocytes and regulation of NF- $\kappa$ B activity can tip the balance between cell survival and death.

CpG ODN induces the sustained activation of NF- $\kappa$ B and degradation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in human and murine B cells [146, 168]. The maturation of CpG DNA-containing endosomes results in the formation of reactive oxygen species (ROS) [139]. ROS are generated within 5 min of treatment with CpG DNA in B cells, and are essential for degradation of I $\kappa$ B $\alpha$  and I- $\kappa$ B $\beta$  [139] and activation of NF- $\kappa$ B. In human B cells, NF- $\kappa$ B was activated within one hour and the NF- $\kappa$ B activation was observed up to 18 hours of exposure to CpG ODN [129]. The sustained activation of NF- $\kappa$ B induced by TLR9 ligand has been implicated in the rescue of primary mouse splenic B cells from spontaneous apoptosis [146] and immature B cells from BCR-mediated apoptosis [168]. TLR9-induced NF- $\kappa$ B activation promotes survival of B cells.

#### ***1.8.4 Activation of Mitogen Activated Protein kinases (MAP kinases)***

CpG DNA activates mitogen activated protein kinases (MAPKs), p38, c-Jun NH2 terminal kinase (JNK) and extracellular-signal regulated kinase (ERK). MAP kinases p38 and JNK, but not ERK, are activated in murine and human B cells [129, 169, 170]. However, ERK is activated in macrophages in response to CpG stimulation [171]. JNK and p38 induce the phosphorylation of c-jun and activating transcription factor 2 (ATF-2) and activating protein 1 (AP-1) [129, 169]. In murine B cells, CpG-induced p38 and AP-1 activation is essential for IL-6 and IgM secretion [169].

### ***1.8.5 Transcription and translation***

Activation of MAP kinases by TLR9 ligand leads to induction of various transcription factors in B cells. These include activating protein -1 (AP-1), activating transcription factor-2 (ATF2) and cyclic AMP response element binding protein (CREB) [138, 169]. Protein levels of pro-survival genes like c-myc and Bcl-xl are upregulated between 1 and 6 hours of treatment with TLR9 ligand [128, 172], while *bcl-2*, *myn*, *myb* and *bax* mRNA levels remain unchanged [172]. Upregulation of transcription factors and survival genes by CpG-containing DNA rescues mature splenic B cells from apoptosis and induces the secretion of cytokines like IL-12, which lead to a Th-1 type immune response.

### **1.9 Cellular Effects of CpG DNA in B cells**

TLR9 ligand, CpG DNA, promotes polyclonal proliferation of B cells. Resting splenic B cells treated with CpG DNA are rescued from spontaneous apoptosis [128]. CpG ODN inhibits BCR-mediated apoptosis in WEHI 231, an immature B cell line [168, 172]. B cells that are activated through TLR9 also become sensitive to antigen stimulation and accelerate antigen-specific immune response [114]. CpG DNA treated B cells upregulate co-stimulatory molecules like MHC class II, CD80, CD86, CD40 and CD54 [129, 173]. Hence CpG treatment can activate B cells to undergo polyclonal proliferation in the absence of antigen, antigen-specific proliferation in the presence of antigen, and differentiation into antibody secreting cells

The cellular response to CpG DNA is characterized by the secretion of pro-inflammatory, Th-1 biased cytokines, like interleukin- (IL) 6, IL-12, tumor necrosis factor (TNF)- $\alpha$  and

interferon (IFN)- $\gamma$  [174]. CpG DNA stimulates secretion of IL-6 and IL-12 from murine splenic B cells [174]. IL-6 secretion in B cells depends on the ROS generated by CpG DNA and is sensitive to antioxidants [175]. While IL-6 has been shown to be important in B cell growth and differentiation, neutralizing it with antibodies does not affect CpG DNA induced B cell proliferation. However, CpG DNA-induced IL-6 is important for differentiation of B cells into IgM-secreting plasma cells [175]. In addition to pro-inflammatory cytokines like IL-6 and IL-12, B cells also secrete anti-inflammatory cytokine, IL-10 [176]. IL-10 counteracts with IL-12 secreted by B cells and macrophages in response to CpG DNA. Immature mouse B cell line, WEHI 231 has also been reported to secrete TNF- $\alpha$  when stimulated with CpG ODN [170]. pDCs stimulated by CpG DNA are the primary sources of type I interferons ( $\alpha$  and  $\beta$ ) [177]. pDCs from mice and humans and monocytes from mice also secrete TNF- $\alpha$ , IL-6 and IL-12 [178] in response to TLR9 ligand. IL-12 and IFN- $\alpha$  stimulate NK cells and T cells to release IFN- $\gamma$ , which can in turn activate B cells [179]. Thus, CpG DNA induces B cells and pDCs to secrete pro-inflammatory cytokines.

In addition to provoking the release of Th-1 skewing cytokines, CpG ODN also promote the differentiation of B cells to plasma cells, secreting antigen-specific antibodies with Th1- like isotypes, IgG<sub>2a</sub>, IgG<sub>2b</sub> and IgG<sub>3</sub> [173]. T-bet (T-box expressed in T cells) that regulates transcriptional activation of Th-1- associated genetic programs is upregulated by CpG DNA. The upregulation of T-bet actively represses IgG<sub>1</sub> and IgE switching in B cells [180].

Taken together, signaling through TLR9 has strong impact on B cell mediated immune response. CpG DNA rescues B cells from apoptosis and induces polyclonal and antigen-specific B cell proliferation. It leads to the secretion of pro-inflammatory cytokines and the differentiation of B cells to plasma cells secreting Th-1 isotype of antibodies, inducing a predominantly Th-1 biased immune response. Hence TLR9 ligand, CpG-containing oligonucleotides, play important roles in activating humoral immune response.

### **1.10 Differential effects of CpG DNA on B cell subpopulations**

TLR9 is differentially expressed and activated in subsets of B lymphocytes. Naïve human B cells express constitutively lower levels of *tlr9* mRNA compared to CD27<sup>+</sup> memory B cells [181]. Both human naïve and memory B cells undergo proliferation with CpG DNA treatment, however, naïve B cells require the presence of antigen or BCR stimulation along with CpG DNA while memory B cells do not need BCR stimulation to differentiate to plasma cells [181]. TLR9 is also expressed in higher levels in activated human B cells. Human B cells that are stimulated with anti-Ig or anti-CD40 antibodies increase expression of TLR9 [182]. Also, transformed human B cell lines like Epstein Barr virus (EBV)-transformed cell lines, Burkitt lymphoma and follicular lymphoma cell lines express constitutively higher levels of TLR9 mRNA compared to resting untransformed B cells [182]. The TLR9 expression profile of different mouse B cell populations has not been well characterized.

Malignant B cells differ in their responses to CpG ODN. Malignant B cells can upregulate the expression levels of costimulatory molecules like CD40, CD54, CD80,

CD86 and MHC class II in response to TLR9 ligand, but to levels different from primary B cells [183]. B-cell chronic lymphocytic leukemia (B-CLL) and marginal zone lymphomas are sensitive and activated strongly by CpG ODN, while plasmacytomas are unresponsive [184]. Follicular lymphomas, mantle cell lymphomas and large cell lymphomas show intermediate responses. While CpG DNA induces proliferation of malignant B cells, their response is generally weaker than that of primary B cells and is accompanied by an increase in apoptosis in some cases [184], suggesting a different role for TLR9 in malignant B cells.

### **1.11 Role of TLRs in activation induced cell death**

Toll-like receptors are arguably the best-studied innate immune receptors. Signaling by the TLRs can induce apoptosis, yet this remains poorly characterized. While the physiological relevance of TLRs as death receptors remains unclear, it is speculated that apoptosis of activated immune cells limits their lifespan and controls the duration of the inflammatory response, preventing overactive inflammatory responses.

Aliprantis et al., [185] first reported that activation of THP-1 cells, a monocytic cell line, with bacterial lipoproteins (BLPs) through TLR2 induced apoptosis. Introducing human TLR2 into an epithelial cell line, HEK293, by transfection induces apoptosis upon stimulation with BLPs. Fas associated death domain protein (FADD) was shown to be recruited to the death domain in MyD88 upon BLP stimulation. This activates caspase 8 and leads to apoptosis [186]. The 19-kDa *Mycobacterium tuberculosis* protein, p19 [187], and lipoproteins from *Mycoplasma fermentas* [188] can also induce apoptosis by

signaling through TLR2. LPS through TLR4 [189], TLR-7 agonist imiquimod [190], and synthetic double stranded RNA (dsRNA) through TLR3 have also been shown to signal for apoptosis [191], especially in stimulated cells.

TLR9 has been shown to be a potential death receptor. Oligonucleotides (ODNs) containing unmethylated CpG motifs rapidly induce apoptosis in MOLT-4 and Jurkat E6 T leukemia cells when introduced intracytoplasmically [192, 193]. A mouse prostate cancer cell line, RM-1, undergoes apoptosis when treated with ODNs containing CpG and poly G motifs [194]. Recent studies showed that fibroblasts transfected with human TLR9 become sensitized to apoptosis when treated with TLR9 ligand [195]. Jahrsdorfer et al., have reported that biopsies of patients suffering with B cell chronic lymphocytic leukemia (B-CLLs) undergo apoptosis when treated with either immunostimulatory (IS) or non-IS ODN [196]. This indicates that CpG ODN-induced signaling can lead to apoptosis in stimulated cells and malignant cells. The pro-apoptotic effect of TLR ligands on malignant cells expressing TLRs suggests a therapeutic value of TLRs in cancer treatment.

In this dissertation, I sought to compare and characterize the effect of CpG ODN on CH27 mouse lymphoma B cells and mouse splenic B cells. The murine lymphoma B cell CH27 was generated from the peritoneal cavity of 2<sup>a</sup>4<sup>b</sup> mice that were repeatedly immunized with chicken red blood cells [197]. The CH series of lymphomas is thought to exhibit phenotypic characteristics similar to human B cell chronic lymphocytic leukemia (B-CLL) [198]. Based on the previous studies mentioned above, I hypothesized that CpG



ODN would have differential effects on the B-CLL-like murine lymphoma B cells CH27 and murine splenic B cells. I have also compared the activation status of down stream signaling components in CpG ODN-induced signaling pathways between primary B cells and CH27 lymphoma B cells in order to further understand the underlying mechanism for the differential response of lymphoma B cells and primary B cells to TLR9 ligand. Thus, the work described in this dissertation, compares for the first time, the differential response of lymphoma B cells and primary B cells to TLR9 ligand and further indicates that differences in NF- $\kappa$ B and c-myc activation by TLR9 as the potential mechanism for this differential response.

## **Chapter 2: Differential Responses of Mouse Primary B Lymphocytes and B Lymphoma Cells to Toll-like Receptor 9 Ligand**

### **2.1 Abstract**

Toll-like Receptor 9 (TLR9) recognizes microbial DNA containing unmethylated cytosyl guanosyl (CpG) sequences and induces innate immune responses and facilitates antigen-specific adaptive immunity. Recent studies reported that in addition to stimulating innate immunity, TLR9 ligands induce apoptosis in TLR9 expressing cancer cells. To understand the mechanism for TLR9-induced apoptosis, in this study, we compared the effects of CpG containing oligodeoxynucleotides (CpG ODN) on mouse splenic B cells and a mouse lymphoma B cell line, CH27. CpG ODN stimulated the proliferation of primary B cells and rescued them from spontaneous apoptosis. In contrast, CpG ODN inhibited cell proliferation and induced apoptosis in CH27 lymphoma B cells in a sequence-specific, TLR9-dependent fashion. The lymphoma B cell apoptosis was accompanied by a decrease in anti-apoptotic protein Bcl-x1. CpG ODN activated NF- $\kappa$ B and increased c-myc protein levels in both primary and lymphoma B cells, although CH27 cells constitutively expressed a high level of c-myc. While the activation effects were robust and sustained in the primary B cells, CpG ODN-triggered NF- $\kappa$ B activation was transient in the lymphoma B cells. Furthermore, NF- $\kappa$ B inhibitor, but not NF- $\kappa$ B activators or MAP kinase inhibitors, inhibited the proliferation of CH27 lymphoma B cells. Our data suggest that the differential effects of CpG DNA on primary and lymphoma B cells occur due to differences in NF- $\kappa$ B activation. The impaired NF- $\kappa$ B

activation in the lymphoma B cells results in deregulation of the balance between NF- $\kappa$ B and c-myc, ultimately inducing apoptosis.

## 2.2 Introduction

Toll-like receptors (TLRs) belong to a family of pattern-recognition receptors [5] that recognize pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharides and DNAs. The TLRs trigger innate immune responses and play a crucial role in limiting the early replication and spread of pathogens during an infection and subsequently activate antigen presenting cells, which leads to the induction of adaptive immune responses that provides long-lasting protection [199].

Toll-like receptor 9 (TLR9) recognizes unmethylated cytosyl guanosyl (CpG) sequences that are present at a higher frequency in the genomes of prokaryotes than that of eukaryotes [114, 200]. TLR9 is primarily expressed by B cells and plasmacytoid dendritic cells (pDCs) in humans [201] and by macrophages and myeloid dendritic cells (mDCs) in addition to B cells and pDCs in mice [120]. TLR9 binds its ligand in endocytic vesicles, which recruits the adaptor protein MyD88 [136] and leads to the formation of TLR-MyD88-IRAK4-IRAK1-TRAF6 signaling complex [92]. IRAK1 recruits and phosphorylates tumor-necrosis-factor receptor-associated factor 6 (TRAF6) [143]. TRAF6 activates tumor growth factor- $\beta$ -activated protein kinase 1 (TAK1) [144] that activates I $\kappa$ B kinase complex (IKK), which leads the activation of nuclear factor (NF)  $\kappa$ B. TAK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is involved in the activation of MAP kinases JNK, p38 and ERK [145]. MAP kinase p38 and JNK, but not ERK were reported to be activated in B cells treated with CpG ODN [169] and contribute to the production of cytokines like IL-6 [175], IL-10 and IL-12 [176]. Treating B cells with oligodeoxynucleotides containing

unmethylated CpG motifs (CpG ODNs) leads to upregulation of several transcription factors including NF- $\kappa$ B, c-myc, AP-1 and cAMP-responsive element-binding protein (CREB) [129, 170] and results in a strong Th-1 biased immune response with secretion of pro-inflammatory cytokines like IL-6 and IL-12 [174].

CpG DNA rescues mature naive B cells from undergoing spontaneous apoptosis and promotes polyclonal B cell proliferation in culture [128]. This anti-apoptotic effect is attributed to TLR9-induced sustained activation of NF- $\kappa$ B and expression of c-myc and Bcl-xl proteins that block the decrease of mitochondrial membrane potential [128, 146, 172]. Signaling through TLRs has also been shown to induce apoptosis. Bacterial lipoproteins signaling through TLR2, LPS through TLR4, and dsRNA through TLR3 have been shown to induce apoptosis especially in stimulated cells [185, 191, 202]. TLR-mediated apoptosis of activated cells has been suggested as a crucial feedback mechanism that down regulates TLR-induced immune cell activation and prevents an overactive inflammatory response that could lead to autoimmune diseases and disease pathologies like sepsis and airway inflammation [203]. The mechanism for TLR-induced apoptosis remains to be elucidated.

Bacterial DNA has been used as adjuvant for generating effective antibody responses in animals long before the discovery of its receptor, TLR9. Recently gained knowledge on the immunological functions of TLR9 uncovers the potential of CpG ODN as a human vaccine adjuvant and as therapeutics for cancer and autoimmune diseases [126]. It has been reported that CpG ODN either alone or in combination with other therapies like

chemotherapy and monoclonal antibodies induces tumor regression of lung and skin malignancies [126]. In murine models of T cell lymphoma and cervical carcinoma, treatment with CpG DNA induced tumor regression, which was shown to be the result of T cell or NK cell dependent lysis of tumor cells [204, 205]. B cell lymphomas are unique in that they could express TLR9 and directly respond to CpG DNA. A recent report showed that CpG ODNs sensitized B cell chronic lymphocytic leukemia cells and fibroblasts transfected with TLR9 for apoptosis [195, 196]. These studies suggest double effects of TLR9 ligand on cancer cells that express TLR9, namely stimulating immune response against cancer cells and inducing apoptosis of cancer cells.

In this study, we compared the effects of CpG ODN on a B lymphoma cell line CH27 and mouse splenic B cells. We showed that CpG ODN induced apoptosis of the B cell lymphoma line, while stimulating proliferation and inhibiting spontaneous apoptosis of naïve splenic B cells in culture. This effect is TLR9-dependent, as a variant of the lymphoma line that did not express TLR9 did not undergo apoptosis in response to CpG ODN. By comparing the downstream signaling components activated by TLR9, we found that CpG ODN induced an imbalanced activation of NF- $\kappa$ B and c-myc in the lymphoma B cells, a potential mechanism leading to apoptosis.

## **2.3 Materials and Methods**

### **Oligonucleotides**

Nuclease-resistant phosphorothioate oligodeoxynucleotides (ODNs) – biotinylated (B) and unbiotinylated – were purchased from Integrated DNA Technologies (Coralville, IA). The sequence of CpG DNA used was 5' TCCATGAC**CG**TTCTGAC**CG**TT 3', and this CpG ODN (1826) is optimal for activating mouse B cells [114]. The control non-CpG DNA referred to as GpC DNA was 5' TCCATGAG**C**TTCTGAG**C**TT 3'. Polymyxin B (Sigma Aldrich, St.Louis, MO) was used to eliminate the possibility of LPS contamination in the CpG ODN samples.

### **Mice, cells and cell culture**

The mouse B cell lymphoma, CH27, generated and characterized by Haughton et al, [206] is an H-2<sup>k</sup>, IgM<sup>+</sup>, Fc $\gamma$ RIIB1<sup>-</sup> cell line. CH27 B lymphoma cells were cultured at 37°C in DMEM supplemented as described previously [207] and containing 15% FBS.

BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Frederick, MD). To obtain splenic B cells, single-cell suspensions of the splenocytes were prepared, and then subjected to density gradient centrifugation in Histopaque-1119 (Sigma, St. Louis, MO) at 2300 g for 15 min. The lymphocyte-enriched fraction was harvested and washed. The B cells were isolated by specific depletion of T cells using anti-Thy 1.2 antibody and guinea pig complement. The B cells were washed and resuspended in medium.

## **Antibodies and reagents**

Guinea-pig complement and Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY). Thy 1.2-specific monoclonal antibody (mAb) was obtained from PharMingen (San Diego, CA). Rabbit mAb against cleaved caspase 3 (5A1) and rabbit polyclonal antibodies against Bcl-xl, pJNK, pERK, pp38 were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against p65 subunit of NF- $\kappa$ B and I $\kappa$ B and goat anti-Btk were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). AlexaFluor-546 conjugated goat anti-rabbit IgG, AlexaFluor-546 conjugated streptavidin and SYTO-green were from Molecular Probes, Oregon. FITC-Fab fragments of goat anti-mouse IgM were from Jackson Immuno Research (West Grove, PA). 1D4B, a mAb specific for lysosomal associated membrane glycoprotein-1 (LAMP-1) was obtained from hybridomas purchased from ATCC (Rockville, MD). Rabbit anti-mouse c-myc antibody, anti-phosphotyrosine mAb (4G10) and HRP-conjugated goat anti-rabbit IgG antibody were purchased from Upstate (Lake Placid, NY). Anti-Bax mAb (6A7) was from BD Biosciences (San Diego, CA). Anti-alpha tubulin mAb (TU-01) was obtained from Zymed Laboratories (San Francisco, CA). The MAP kinase inhibitors SB203580, SP600125 and PD98059 and NF- $\kappa$ B activation inhibitor, 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline were from EMD Biosciences (San Diego, CA). LPS from *Escherichia coli*, Ionomycin and phorbol myristate acetate (PMA) were purchased from Sigma Aldrich (St.Louis, MO).



### **Cell proliferation assay**

CH27 B cell lymphomas ( $1 \times 10^5$  cells/well) and splenic B cells from BALB/c and C57BL6 mice ( $5 \times 10^5$  cells/well) were placed in 96-well plates and treated with varying concentrations of CpG ODN or control GpC ODN in the presence or absence of inhibitors, for 66 hours.  $1\mu\text{Ci } ^3\text{H}$ - thymidine (MP Biomedicals, Irvine, CA) was added to each well for the last 18 hours of incubation. Cells were harvested and cell associated radiation was counted using a scintillation counter.

### **RT-PCR analyses of *tlr9* mRNA**

Total cellular mRNA was isolated from two different clones of CH27 and splenic B and T cells by Trizol (Invitrogen) using a manufacturer recommended protocol. cDNA were generated from mRNA by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and amplified using *tlr9* specific primers using Taq DNA polymerase (Invitrogen) with 25 cycles. Cycling conditions were  $94^\circ\text{C}$  for 30 sec,  $55^\circ\text{C}$  for 30 sec, and  $68^\circ\text{C}$  for 1 min.  $\beta$ -tubulin gene was amplified as a control, with 25 cycles with the cycling condition as  $94^\circ\text{C}$  for 30 sec,  $56^\circ\text{C}$  for 30 sec, and  $68^\circ\text{C}$  for 1 min. *tlr9*-specific primers were 5'-GCACAGGAGCGGTGAAGGT-3' and 5'-GCAGGGG TGCTCAGTGGAG-3', and  $\beta$ -tubulin-specific primers were 5'-TGGAATCCTGTGGCATCCA-3' and 5'-TAACAGTCCGCCTAGAAGCA-3'.

### **Apoptosis assay**

CH27 lymphomas ( $1 \times 10^5$  cells/ml) and splenic B cells ( $4 \times 10^5$  cells/ml) were incubated in the absence or with 1 or 10  $\mu\text{g/ml}$  of GpC or CpG ODN for 24 or 48 hours. Cellular apoptosis and necrosis were detected using the Vybrant Apoptosis detection kit from

Molecular Probes, Oregon. Briefly,  $1 \times 10^5$  cells were harvested, washed with PBS and resuspended in Annexin Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5mM  $\text{CaCl}_2$ , pH 7.4). Cells were then stained with AlexaFluor 488-conjugated Annexin V and Propidium Iodide for 20 min at room temperature. After incubation 400  $\mu\text{l}$  Annexin Binding Buffer was added and the cells were immediately analyzed by flow cytometry using the FACSCalibur (Becton Dickinson, San Jose, CA). To test the effect of MAP kinase and NF- $\kappa\text{B}$  inhibitors, the cells were grown with the ODN and inhibitors for 24 and 48 hours, harvested and processed as detailed above.

### **Western blot analysis**

CH27 lymphomas ( $2 \times 10^6$  cells/ml) and splenic B cells ( $5 \times 10^6$  cells/ml) were incubated with medium alone or 10 $\mu\text{g/ml}$  ODN at 37°C for various times. Reactions were terminated by pelleting the cells at 4°C, followed by lysis of cells in 1% Nonidet P-40 lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF and protease-inhibitor cocktail) for 30 min at 4°C. Equal concentrations of lysates were subjected to SDS-PAGE and blotted with antibodies to either cleaved caspase 3, I $\kappa\text{B}\alpha$ , Bcl-xl, Bax, c-myc or phospho MAP kinases, followed by HRP-conjugated goat anti-rabbit IgG antibodies. The blots were then stripped and reblotted with anti-tubulin antibodies and an HRP-conjugated secondary antibody as loading controls. CH27 lymphomas ( $2 \times 10^6$  cells/ml) were incubated with medium alone or 1 $\mu\text{g/ml}$  LPS at 37°C for various times and lysates were obtained as described above. These were subject to SDS-PAGE and probed with anti-I $\kappa\text{B}\alpha$  antibody. Densitometry analysis of the blots was carried out and normalized to the density of tubulin band in the

same sample. The numbers were plotted and represent the fold induction from the normalized densitometric value of either the positive control or untreated sample.

### **Immunofluorescence microscopy**

Cells ( $1 \times 10^6$ /ml) were incubated with 7  $\mu$ g/ml of ODNs at 37°C for varying lengths of time. Cells were washed with DMEM containing 6 mg/ml of BSA (DMEM-BSA) and adhered onto poly-L-lysine-coated slides (Sigma, St. Louis, MO) for 40 min at 4°C. At the end of the incubation, cells were fixed and permeabilized with cold methanol, and incubated with antibody specific for the p65 subunit of NF- $\kappa$ B and AlexaFluor 546 conjugated goat anti-rabbit IgG. Cells were also incubated with SYTO-green stain for 20 min to stain the nucleus. To visualize BCR and CpG ODN, cells were incubated with 20  $\mu$ g/ml F(ab) fragment of FITC-goat anti-mouse IgM for 10 min, washed with DMEM-BSA and adhered onto poly-L-lysine-coated slides (Sigma, St. Louis, MO) for 40 min at 4°C and then incubated with B-CpG DNA for 30 min at 37 °C. At the end of the incubation, cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.05% saponin, and incubated with antibody specific for LAMP-1 (ID4B) and AlexaFluor 546-conjugated goat anti-rat IgG and AlexaFluor 546-conjugated streptavidin. Cells were mounted with Gel/Mount (Biomedex, Foster City, CA). Slides were analyzed by laser-scanning confocal microscopy (LSM 510, Zeiss) using 100 x oil immersion objective. The same settings on the confocal microscope were used for all experimental conditions. Control experiments where the primary antibody was omitted showed no significant staining signals. To quantify the immunofluorescence data, cells in five images randomly taken from slides of each of three independent experiments were counted for each time

point and condition. The number of cells showing nuclear translocation of p65 subunit of NF- $\kappa$ B was counted and expressed as a percentage of the total number of cells in the images.

## 2.4 Results

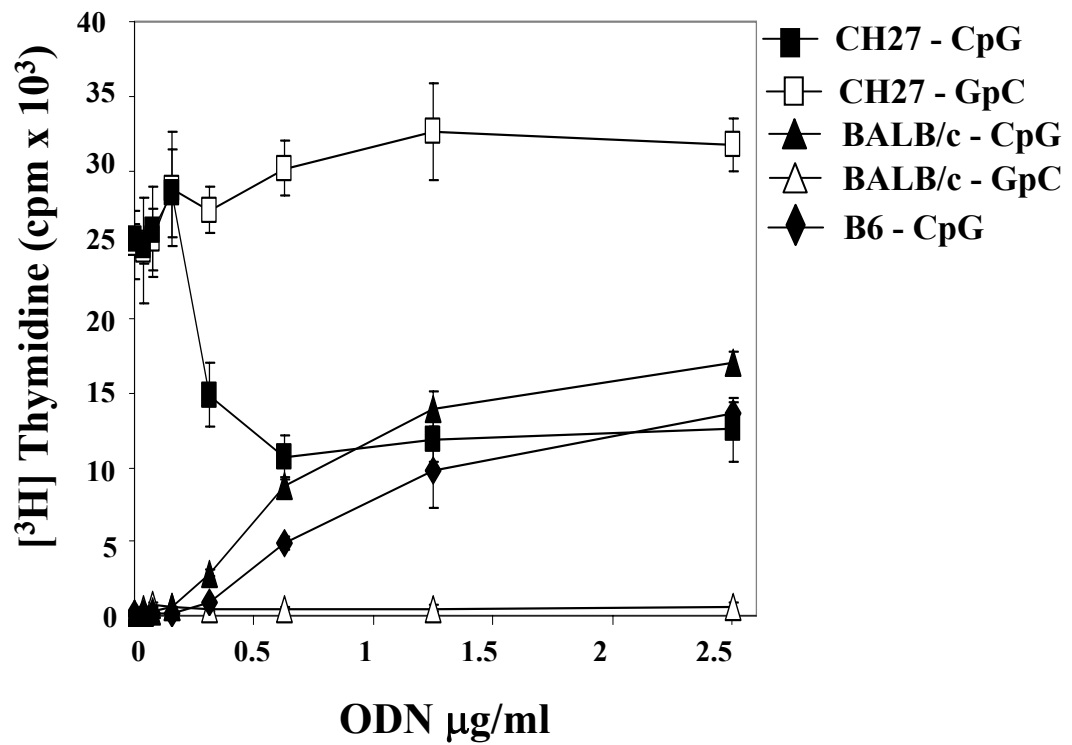
### **Differential effects of CpG DNA on proliferation of B cell lymphoma lines and primary B cells.**

Previous studies have shown that synthetic oligodeoxynucleotides containing unmethylated CpG motif (CpG ODNs) inhibited spontaneous apoptosis of splenic B cells [128] and sensitized human B cell chronic lymphocytic leukemia cells for apoptosis [196]. We sought to compare the effects of CpG ODN on the proliferation of mouse lymphoma B cell line CH27 and murine splenic B cells. The CH27 B cell line was established from lymphatic tumors of mice that were repeatedly immunized with sheep red blood cells [206]. CH27 cells and splenic B cells from BALB/c and C57BL/6 mice were cultured with varying concentrations of CpG ODN for 48 h, and the rate of cell proliferation was detected using [ $^3\text{H}$ ]-thymidine incorporation. An ODN with the CpG motif switched to GpC (GpC ODN) was used as a control. As previously reported, there was a dose dependent increase in proliferation of splenic B cells upon treatment with CpG ODNs, while the control GpC ODN failed to induce the proliferation of splenic B cells (Fig. 2-1A). The splenic B cells from BALB/c and C57BL/6 showed similar increases in the rate of proliferation in response to CpG ODN. Surprisingly, CH27 lymphoma B cells, which constitutively proliferate in culture, reduced their proliferation rate as CpG ODN concentration increased. When cultured in 0.5  $\mu\text{g/ml}$  (0.07  $\mu\text{M}$ ) of CpG ODN, the proliferation rate of CH27 cells was reduced 60%. No further reduction in the proliferation rate of CH27 cells was observed when CpG ODN concentration was over 0.5  $\mu\text{g/ml}$ , suggesting that the inhibitory effect of CpG ODN was saturable. The control GpC ODN did not affect the

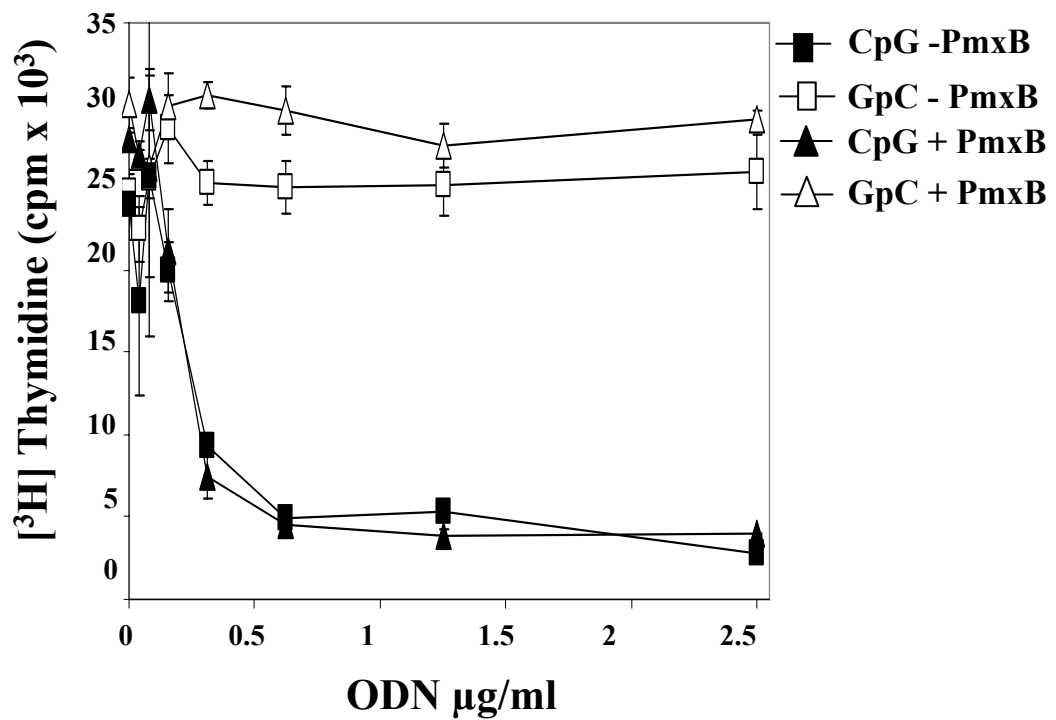
**Figure 2-1. Differential effects of CpG DNA on proliferation of lymphoma B cell line and primary B cells.**

(A) CH27 lymphoma B cell ( $1 \times 10^5$  cells/ml) and splenic B cells from BALB/c and C57B6 mice ( $5 \times 10^5$  cells/ml) were treated with varying concentrations of CpG ODN (filled symbols) or GpC ODN (open symbols) for 66 h. [ $^3\text{H}$ ]- thymidine ( $1 \mu\text{Ci}$ ) was added to each well for the last 18 hours of incubation, cells were harvested and cell associated radioactivity was counted using a scintillation counter. Data represent mean ( $\pm$ S.D.) of triplicate samples. Shown are the representative results from three independent experiments. Squares represent CH27 lymphoma B cells. Triangles represent splenic B cells from BALB/c mice. Diamonds represent splenic B cells from C57BL/6 mice. (B) CH27 lymphoma B cells ( $1 \times 10^5$  cells/ml) were treated with varying concentrations of CpG ODN (filled symbols) or GpC ODN (open symbols) in the presence or absence of polymyxin B for 66 h and the assay was carried out and analyzed as described in (A).

**A**



**B**



proliferation of CH27 cells. These results indicate that CH27 lymphoma B cells respond to CpG ODN differently from splenic B cells.

To eliminate the possibility of lipopolysaccharide (LPS) contamination in the ODNs and ensure the ODN specificity of the anti-proliferatory effect of CpG ODN on CH27 B cell lymphomas, the CH27 B cell lymphomas were treated with Polymyxin B along with the ODNs. Polymyxin B is an antibiotic which sequesters LPS by forming PolymyxinB-LPS complexes, thereby suppressing the biological activity of LPS [208]. The CH27 B cell lymphomas treated with Polymyxin B and ODNs show very similar rate of proliferation compared to cells treated with CpG ODN alone (Fig. 2-1B). This rules out the possibility that the apoptotic effect seen in the B cell lymphomas is due to LPS contamination and reinforces the ODN specificity of the CpG-induced apoptosis.

#### **CpG ODN induces apoptosis in CH27 lymphoma B cells.**

One possible mechanism for inhibiting the proliferation of CH27 lymphoma B cells is the induction of apoptosis. To test this hypothesis, we quantified the percentage of cells undergoing apoptosis by flow cytometry. CH27 lymphoma and splenic B cells from BALB/c mice were treated with different concentrations (1  $\mu$ g/ml or 10  $\mu$ g/ml) of CpG ODN or control GpC ODN for 24 and 48 h. At the end of the incubation, cells were stained with Alexa Fluor 488-conjugated Annexin V and propidium iodide (PI) and analyzed by flow cytometry. Cells that were not stained by either Annexin V or PI were considered as live cells. Annexin V binds to the phosphatidyl serine present on the outer leaflet of the plasma membrane of apoptotic cells and cells stained with Annexin V only

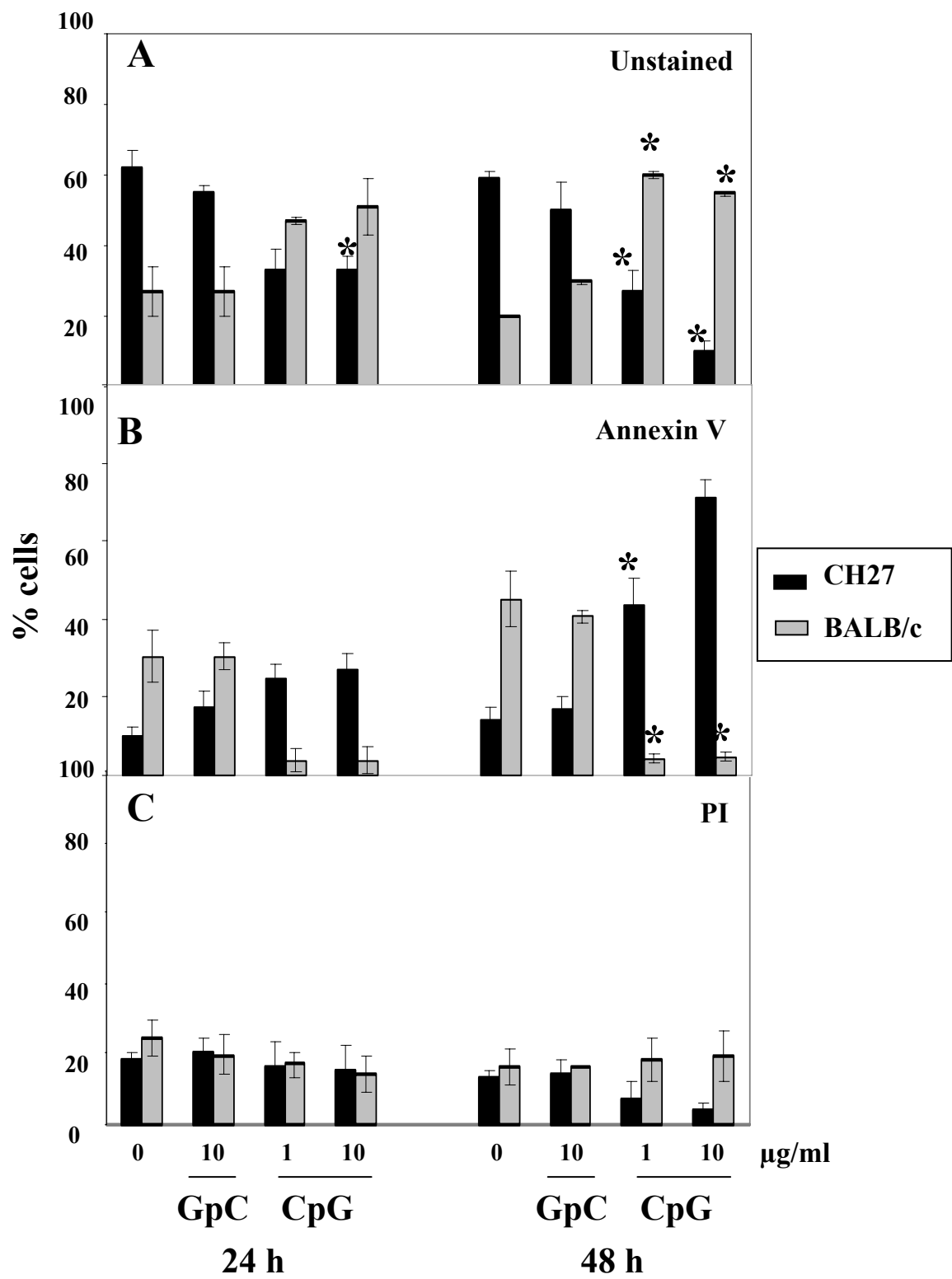


were considered apoptotic cells. PI is impermeant to live cells and apoptotic cells, but binds to the nucleic acids of necrotic and dead cells [209, 210]. Splenic B cells that were untreated or treated with the control GpC ODN had 30% unstained live cells. CpG ODN treatment increased the percentage of live cells to 50% at 24 h and 60% at 48 h (Fig. 2-2A) and reduced the percentage of apoptotic splenic B cells from 50% to less than 10% after 48 h of the treatment (Fig. 2-2B). In contrast, CpG ODN treatment decreased the percentage of live CH27 cells from 60% to 30% after 24 h of the treatment. The percentage of live CH27 cells further declined to less than 20% when the CpG ODN treatment was extended to 48 h (Fig. 2-2A). Significantly, there was a concomitant increase in the percentage of Annexin V positive, apoptotic CH27 cells when treated with CpG ODN. While only 20% of GpC ODN treated cells were apoptotic, CpG ODN increased the percentage of apoptotic CH27 cells to more than 50% at 24 h and up to 80% at 48 h (Fig. 2-2B). There was no significant difference in the percentage of PI-positive CH27 cells or splenic B cells after 24 or 48 h of treatment with CpG ODN, compared to untreated and GpC ODN-treated cells (Fig. 2-2C). Taken together, these results show that while CpG ODN rescued splenic B cells from apoptosis, it induced apoptosis in CH27 lymphoma B cells. GpC ODN that does not activate TLR9 had no significant effect on apoptosis of both CH27 lymphoma and splenic B cells.

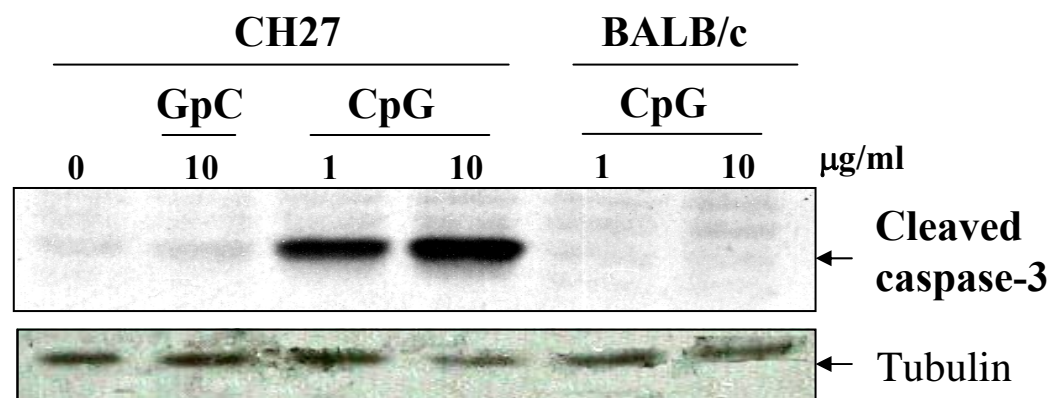
To further confirm CpG ODN-induced apoptosis in CH27 cells, we followed the level of cleaved caspase-3, one of the key executioners of apoptosis. Caspase-3 is activated by the cleavage of its inactive zymogen form (35 kDa) into activated p17 and p12 forms, which is one of the hallmarks of caspase-mediated apoptosis [211]. The generation of cleaved

**Figure 2-2. CpG induces apoptosis of B cell lymphoma line.**

CH27 B cell lymphomas (black bars) and splenic B cells (grey bars) were incubated with varying concentrations of GpC and CpG ODNs (1  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$ ) for 24 and 48 h. The cells were harvested, stained with Propidium Iodide (PI) and Annexin V and 10,000 cells were analyzed by flow cytometry. The percentages of unstained cells (A), Annexin V positive cells (B) and PI positive necrotic cells (C) were plotted. Shown are the mean  $\pm$  S.D. from three independent experiments. \* refers to a statistically significant difference in percentages of cells compared to GpC ODN treated control samples ( $P < 0.02$ ). (D) CH27 lymphoma B cell ( $2 \times 10^6$  cells/ml) and splenic B cells ( $5 \times 10^6$  cells/ml) were incubated with or without 1 or 10  $\mu\text{g/ml}$  CpG for 24 h. The cells were lysed, and the lysates were subjected to SDS-PAGE and Western blotting, probing with antibodies specific for cleaved caspase-3. Equal amount (20  $\mu\text{g}$  proteins/lane) of cell lysates were loaded. Blots were stripped and probed for tubulin as loading controls. Shown are the representative results from four independent experiments.



**D**



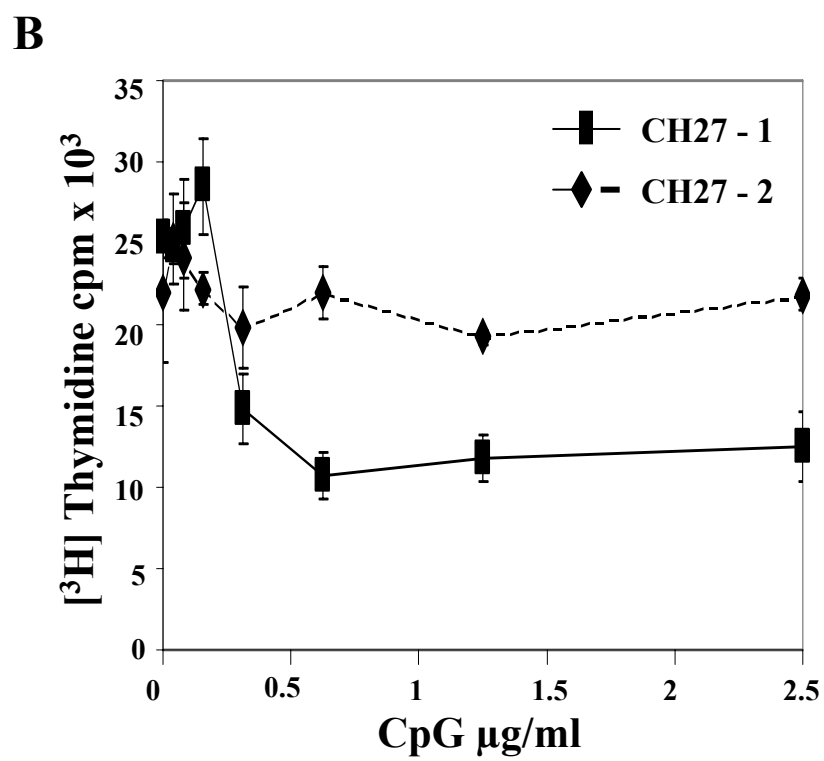
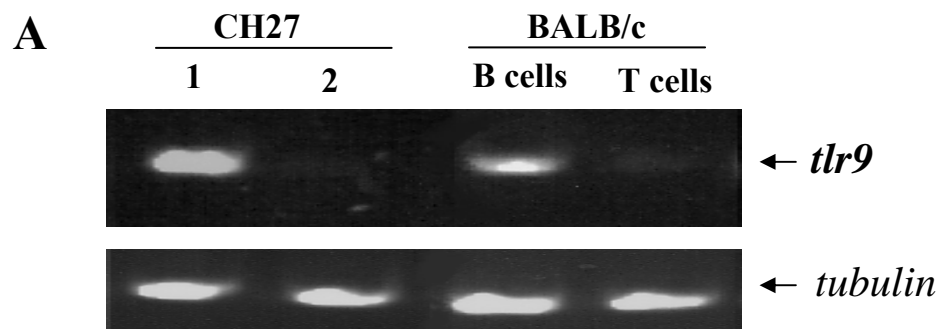
caspase-3 was followed by Western blots. CH27 and BALB/c cells were treated for 24 h with CpG or control GpC ODN and lysed, and the lysates were subjected to SDS-PAGE and Western blot, and probed with an antibody specific for cleaved caspase-3. CpG ODN treatment for 24 h resulted in increased levels of cleaved caspase-3 in CH27 lymphoma B cells (Fig. 2-2D). In contrast, cleaved caspase-3 was undetectable in splenic B cells under similar treatments. The control GpC ODN did not increase the levels of cleaved caspase-3 in CH27 cells (Fig. 2-2D). This confirms and extends our cytometry results that CH27 lymphoma B cells undergo caspase-dependent apoptosis in response to CpG ODN.

**The anti-proliferation and pro-apoptotic effects of CpG ODN on CH27 lymphoma B cells are dependent on TLR9.**

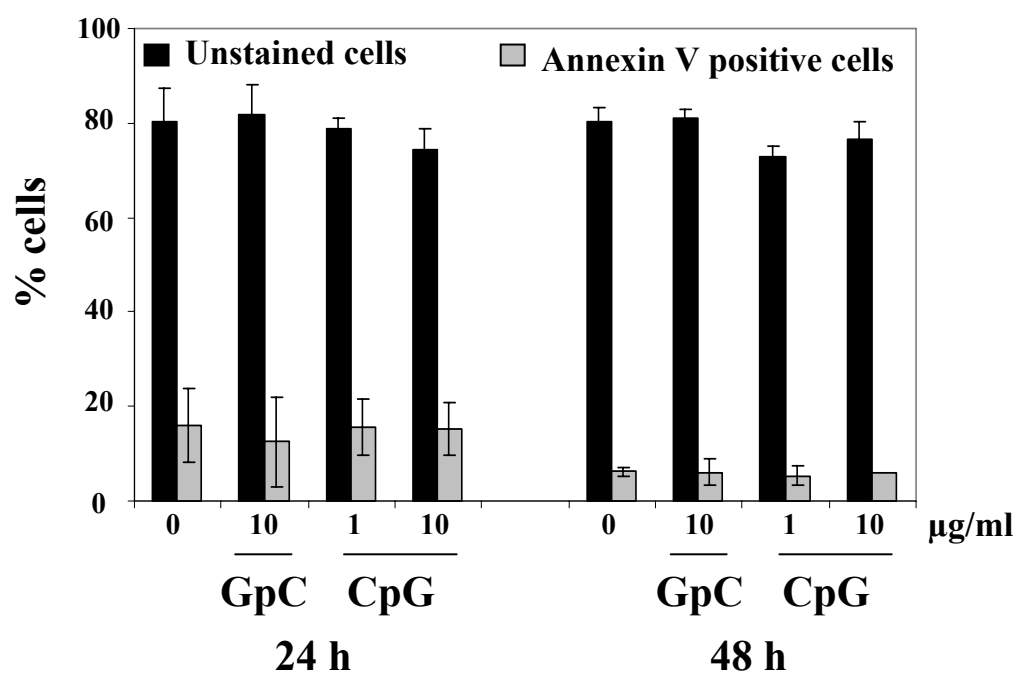
To test whether the inhibition of CH27 cell proliferation and induction of CH27 cell apoptosis by CpG ODN are dependent on TLR9, we compared the effects of CpG ODN on the proliferation of two different clones of CH27 cells that were selected by limiting dilution. Their *tlr9* mRNA expression levels were determined by RT-PCR. CH27 clone 1 and mouse splenic B cells expressed significant levels of *tlr9* mRNA. However, *tlr9* mRNA was undetectable in CH27 clone 2 and splenic T cells (Fig. 2-3A). In contrast to the anti-proliferation and pro-apoptotic effects of CpG ODN on CH27 clone 1 that expressed *tlr9* mRNA, CpG ODN neither altered the proliferation rate (Fig. 2-3B) nor induced apoptosis (Fig. 2-3C) of CH27 clone 2 that did not express *tlr9* mRNA. This suggests that CpG ODN inhibits the proliferation and induces apoptosis of CH27 clone 1 through TLR9.

**Figure 2-3. The anti-proliferation and anti-apoptotic effect of CpG ODN on CH27 lymphoma B cells are mediated through TLR9.**

(A) Total cellular mRNA was isolated from two CH27 lymphoma clones and splenic B (B) and T (T) cells from BALB/c mice using Trizol. TLR 9 mRNA was amplified by RT-PCR. Tubulin mRNA was also amplified as a control. (B) CH27 lymphoma B cells clone 1 and 2 ( $1 \times 10^5$  cells/ml) were treated with varying concentrations of CpG ODN for 66 h. The cell proliferation was analyzed by [ $^3\text{H}$ ]- thymidine incorporation as described in Figure 2-1. Data represent mean ( $\pm$ S.D.) of triplicate samples. Shown are the representative results from three independent experiments. (C) CH27 lymphoma B cells clone 2 was treated as described in Figure 2-2 and the percentage of live and apoptotic cells were determined. Shown is the average ( $\pm$ S.D.) of three independent experiments.



**C**



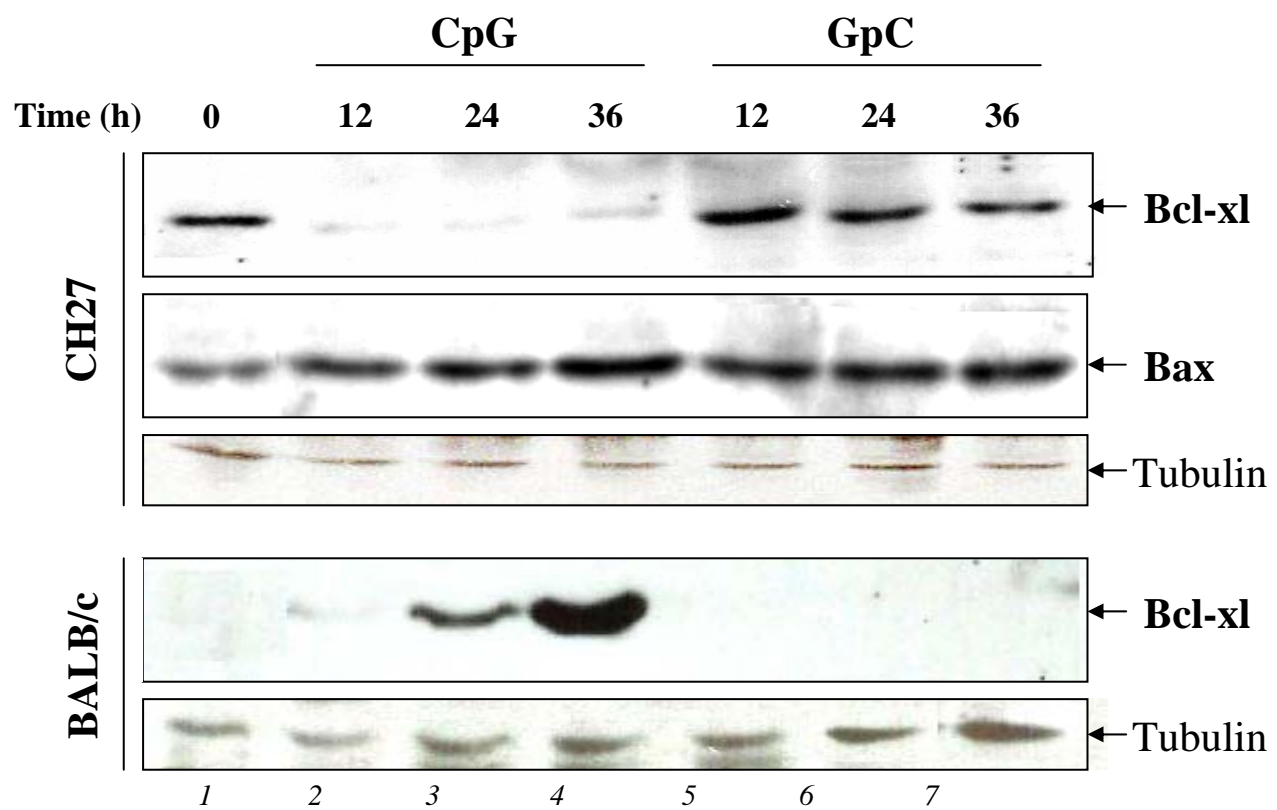


**Differential effects of CpG ODN on the expression of apoptosis regulatory proteins in CH27 lymphoma and splenic B cells.**

The Bcl-2 family of proteins is critical in maintaining mitochondrial integrity and regulating apoptosis. Bcl-xl and Bax are two major apoptotic regulators in B cells [212], with Bcl-xl being an anti-apoptotic and Bax, a pro-apoptotic member of the Bcl-2 family. Stimulation with CpG DNA has been reported to increase Bcl-xl protein levels and rescue splenic B cells from apoptosis [172]. To understand how TLR9 induces the apoptosis of CH27 lymphoma B cells, we compared the effect of CpG ODN on the protein levels of Bcl-xl and Bax in CH27 and splenic B cells by Western blot. CH27 constitutively expressed high levels of Bcl-xl (Fig. 2-4, top panel, lane 1), similar to other lymphoma B cells [213]. CpG ODN-treatment decreased Bcl-xl expression level, and this decrease was observed as early as 12 h of CpG treatment (Fig. 2-4, top panel, lane 2). In contrast, freshly isolated naïve splenic B cells had no detectable level of Bcl-xl (Fig. 2-4, bottom panel, lane 1), and showed an increase in Bcl-xl expression level after 24 and 36 h of CpG treatment (Fig. 2-4, bottom panel, lane 3, 4). CpG ODN treatment did not result in any significant change in Bax protein levels in CH27 B lymphoma cells (Fig. 2-4, top panel, lane 1- 7). This indicates that CpG ODN differentially regulates the protein expression levels of Bcl-xl in B lymphoma CH27 cells and splenic B cells, which could lead to different fates of these two types of B cells.

**Figure 2-4. Differential effects of CpG DNA on the expression levels of Bcl-xl and Bax in lymphoma and primary B cells.**

CH27 clone 1 ( $2 \times 10^6$  cells/ml) and splenic B cells ( $5 \times 10^6$  cells/ml) were cultured in the absence or presence of 10  $\mu$ g/ml CpG or GpC ODN for 12, 24 and 36 h. The cells were lysed and lysates subjected to SDS-PAGE and Western blotting. Blots were probed with anti-Bcl-xl and anti-Bax antibodies followed with HRP-conjugated secondary antibodies. Equal amount (30  $\mu$ g protein/lane) of the cell lysates was loaded. Blots were stripped and probed with antibody against tubulin, which serves as a loading control. Shown are the representative results from four independent experiments



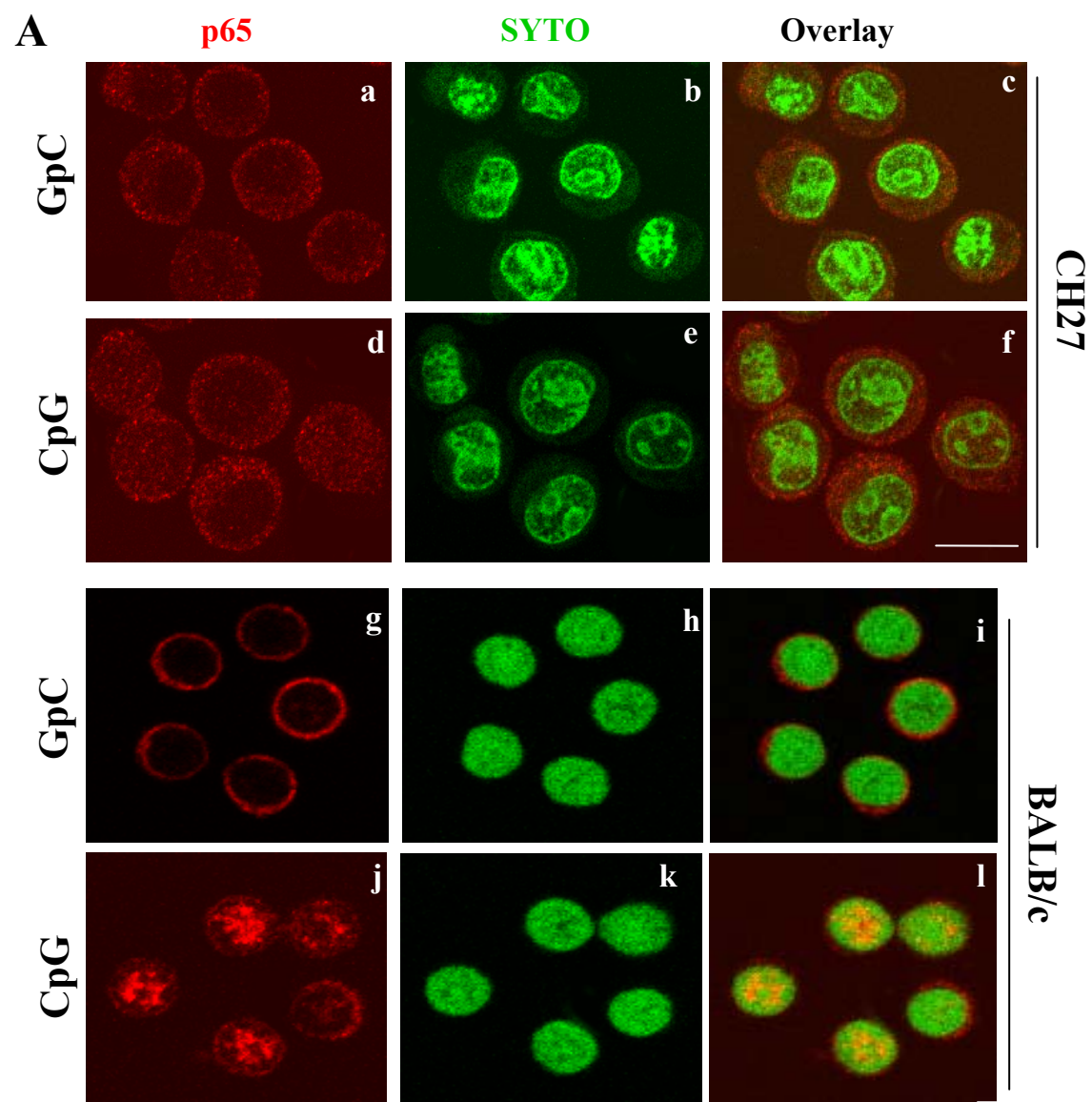
### **Differential effects of CpG ODN on the activation of NF- $\kappa$ B in CH27 lymphoma and splenic B cells.**

NF- $\kappa$ B is the main downstream target of TLR9 signaling pathway [146, 170] and a key regulator of B cell fate. Next, we tested whether CpG ODN induces differential levels of NF- $\kappa$ B activation by following the nuclear translocation of NF- $\kappa$ B p65 subunit and degradation of the inhibitory component of NF- $\kappa$ B, I $\kappa$ B $\alpha$ . The nuclear translocation of NF- $\kappa$ B p65 was followed by immunofluorescence microscopy. CH27 lymphoma and splenic B cells were treated with CpG ODN (7  $\mu$ g/ml) or control GpC ODN for varying lengths of time. At the end of each time point, the cells were fixed, permeabilized, and stained with a mAb specific for the p65 subunit of NF- $\kappa$ B and a DNA dye. There was a minimal and similar basal level of NF- $\kappa$ B p65 in the nuclei of both splenic and CH27 lymphoma B cells. The nuclear staining levels of p65 in both B cell types were increased after 15 and 45 min of treatment with the CpG ODN (Fig. 2-5B). NF- $\kappa$ B p65 staining level in the nuclei of splenic B cells continued increasing with nearly 80% of the splenic B cells exhibiting NF- $\kappa$ B nuclear translocation after 120 min of treatment with CpG ODN (Fig. 2-5Aj – l). However, in the CH27 lymphoma B cells, NF- $\kappa$ B p65 staining level in the nuclei returned to the basal level after 120 min of CpG ODN treatment with only 20% of the cells showing NF- $\kappa$ B nuclear translocation (Fig. 2-5Ad – f).

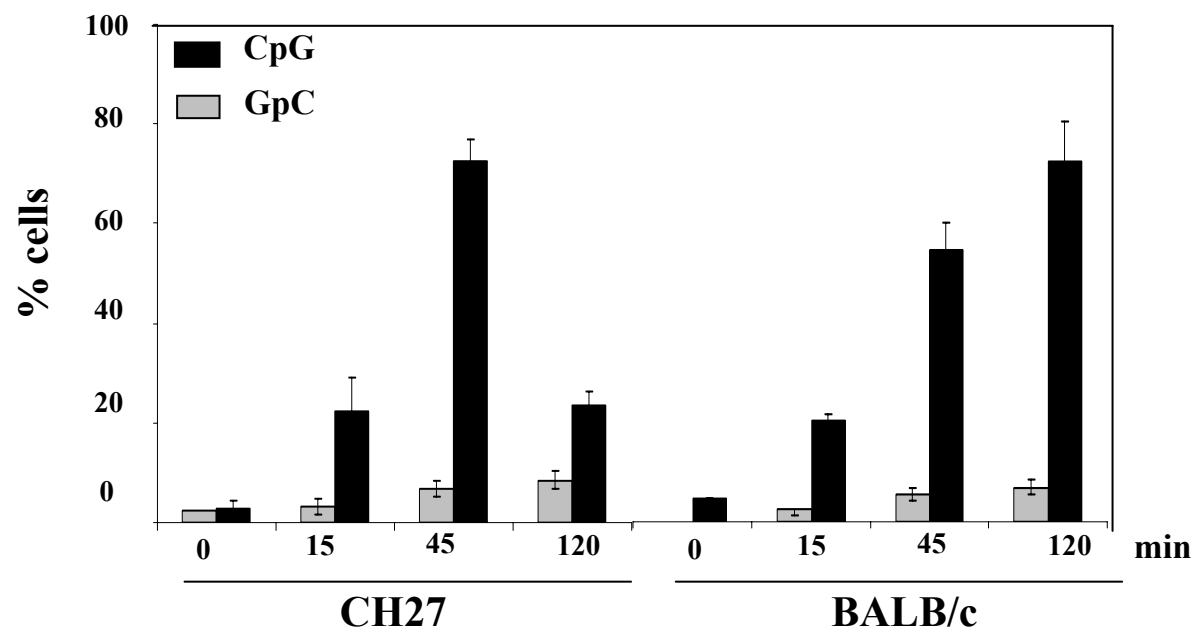
The degradation of the inhibitory component of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , was followed by Western blot. CpG ODN treatment induced a continuous decrease of I $\kappa$ B $\alpha$  in splenic B cells up to 2 h. However, in CH27 lymphoma B cells the same treatment resulted in a transient decrease of I $\kappa$ B $\alpha$  level at 30 min, but returned to basal level by 90 min (Fig. 2-5C, top

**Figure 2-5. Differential effects of CpG DNA on NF- $\kappa$ B activation in lymphoma and primary B cells.**

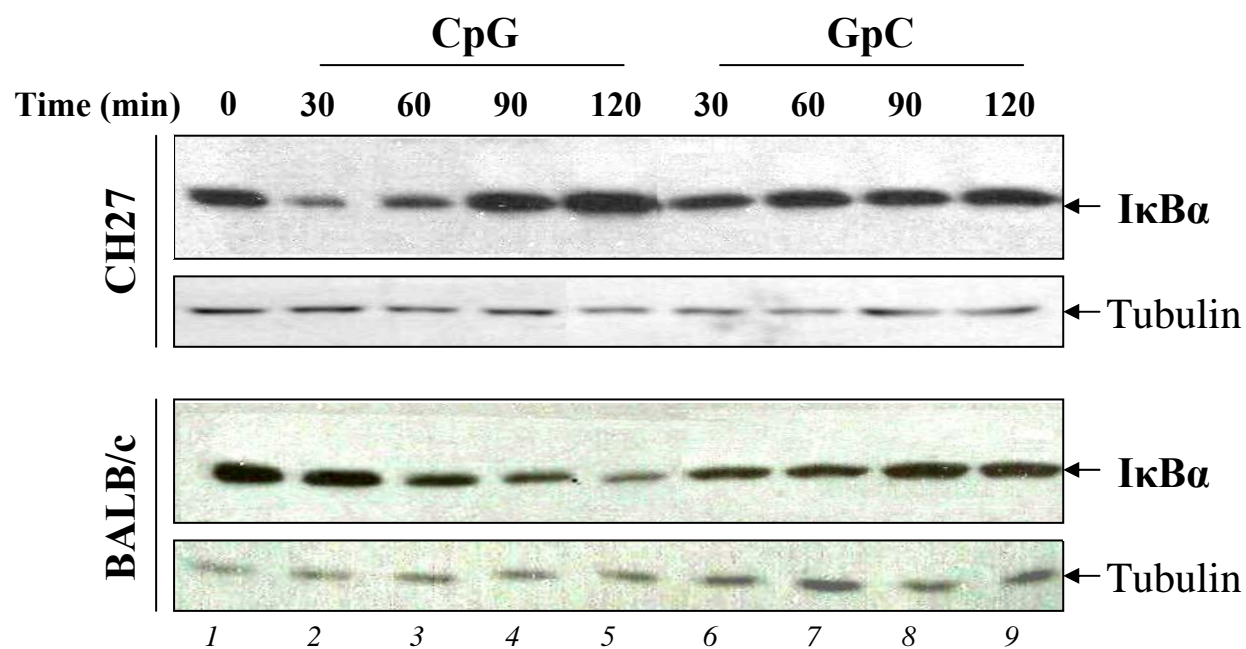
(A) CH27 clone 1 (a – f) and splenic B cells (g – l) ( $1 \times 10^6$  cells) were treated with  $7\mu\text{g/ml}$  CpG or GpC DNA for various times at  $37^\circ\text{C}$ . The cells were then fixed, permeabilized, and incubated with a mAb specific for p65 subunit of NF- $\kappa$ B and Alexa Fluor 546-conjugated secondary antibody. Cells were also incubated with SYTO-green to stain the nucleus. Images were acquired in the middle of the cells using a confocal fluorescence microscope. Shown are representative images of cells after 2 h of exposure to ODNs. Bar,  $10\mu\text{m}$ . (B) Shown is a quantitative analysis of the effect of CpG ODN on translocation of p65 subunit of NF- $\kappa$ B to the nucleus. Hundred cells were counted from five random fields of each time point of three independent experiments. The percentages of CH27 lymphomas and splenic B cells showing translocation of the p65 subunit of NF- $\kappa$ B to the nucleus upon exposure to CpG (black bars) or GpC (grey bars) DNA is plotted. Shown is the mean  $\pm$  S.D. from three independent experiments. (C) CH27 lymphomas clone 1 ( $2 \times 10^6$  cells/ml) and splenic B cells ( $5 \times 10^6$  cells/ml) were treated with  $10\mu\text{g/ml}$  CpG and GpC DNA for various times (30 min to 2 h). After stimulation, cells were lysed and the lysates subjected to SDS-PAGE and Western blot, probing with anti-I $\kappa$ B $\alpha$  antibody and an HRP-conjugated secondary antibody. Equal amounts ( $30\mu\text{g}$  proteins/lane) of cell lysates were loaded. The experiment was repeated five times with similar results.



**B**



**C**



panel, lane 2 - 4). The control GpC ODN did not alter the protein levels of  $\text{I}\kappa\text{B}\alpha$  in either cell type (Fig. 2-5C, lane 6 - 9). These results showed that CpG ODN induced a sustained increase of NF- $\kappa$ B activation in splenic B cells, but only a transient activation of NF- $\kappa$ B in CH27 lymphoma B cells.

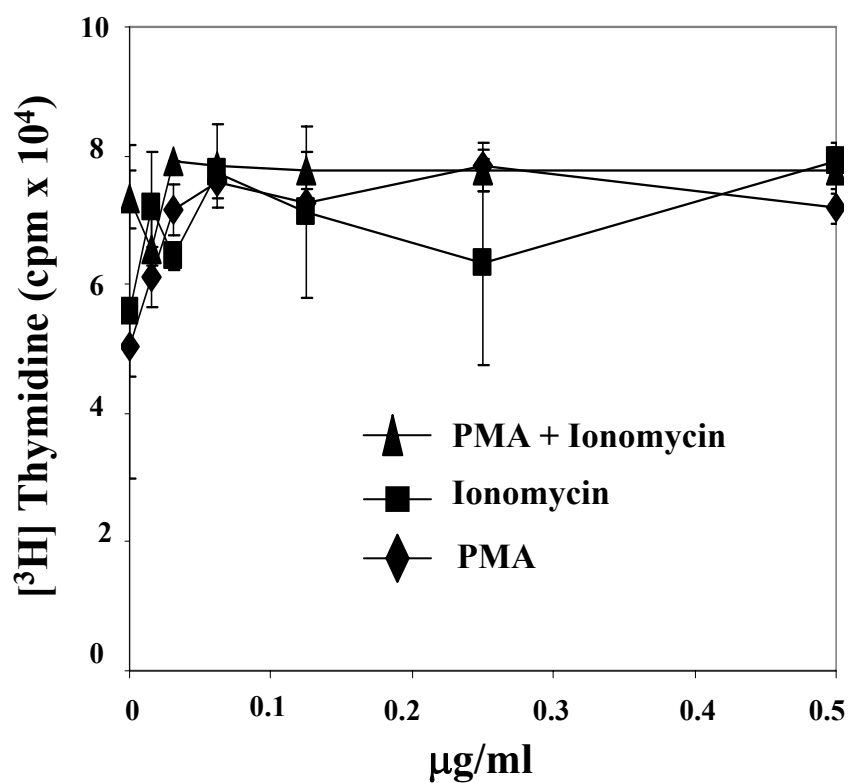
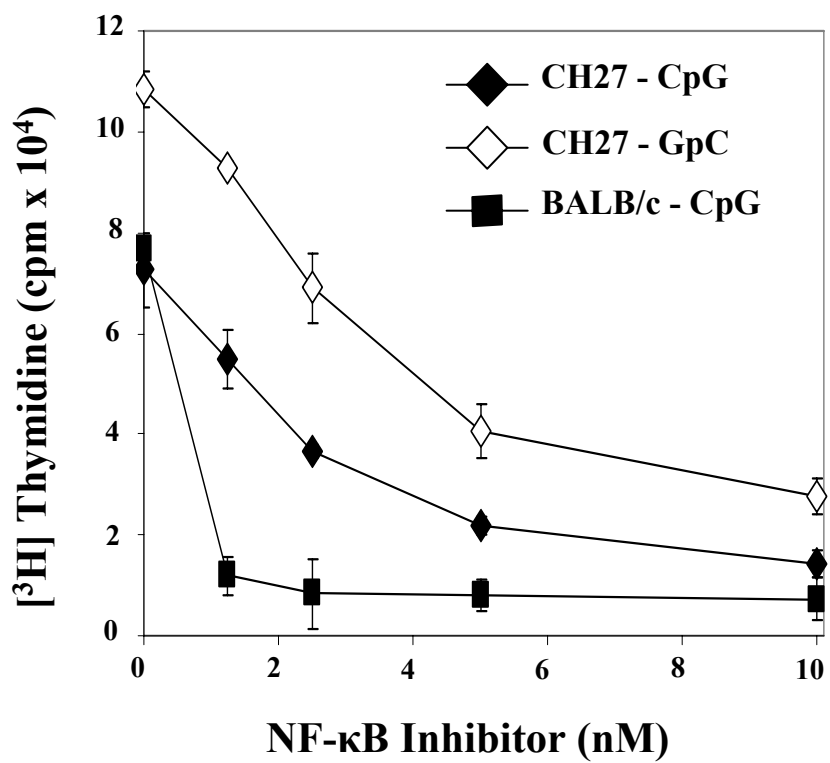
### **Effects of NF- $\kappa$ B activators and inhibitor on the proliferation of CH27 lymphoma B cells and splenic B cells.**

CpG ODN treatment of CH27 induced a transient activation of NF- $\kappa$ B, which was concurrent with a decrease in cell proliferation and an increase in apoptosis. To further test the involvement of NF- $\kappa$ B in CpG ODN-induced apoptosis, we determined the effect of NF- $\kappa$ B activators and inhibitor on the proliferation of the two types of B cells. In B cells upon BCR ligation, protein kinase C functions as a major upstream activator of NF- $\kappa$ B [214]. To activate protein kinase C, we treated CH27 cells with varying concentrations of phorbol myristate acetate (PMA), ionomycin and a combination of the two. The protein kinase C activators slightly increased the proliferation rate of CH27 cells (Fig. 2-6A). A cell permeable quinazilone compound was used as NF- $\kappa$ B inhibitor. This compound has been shown to inhibit NF- $\kappa$ B transcriptional activation in Jurkat cells with no cytotoxicity even at high concentrations [215]. CH27 B cell lymphomas and splenic B cells were treated with varying concentrations of the NF- $\kappa$ B inhibitor in the presence of CpG or control GpC ODN. The combination treatment of CpG ODN and NF- $\kappa$ B inhibitor induced a stronger inhibition of CH27 proliferation than CpG ODN alone (Fig. 2-6B). CpG-induced proliferation of splenic B cells was also drastically inhibited when the cells were treated

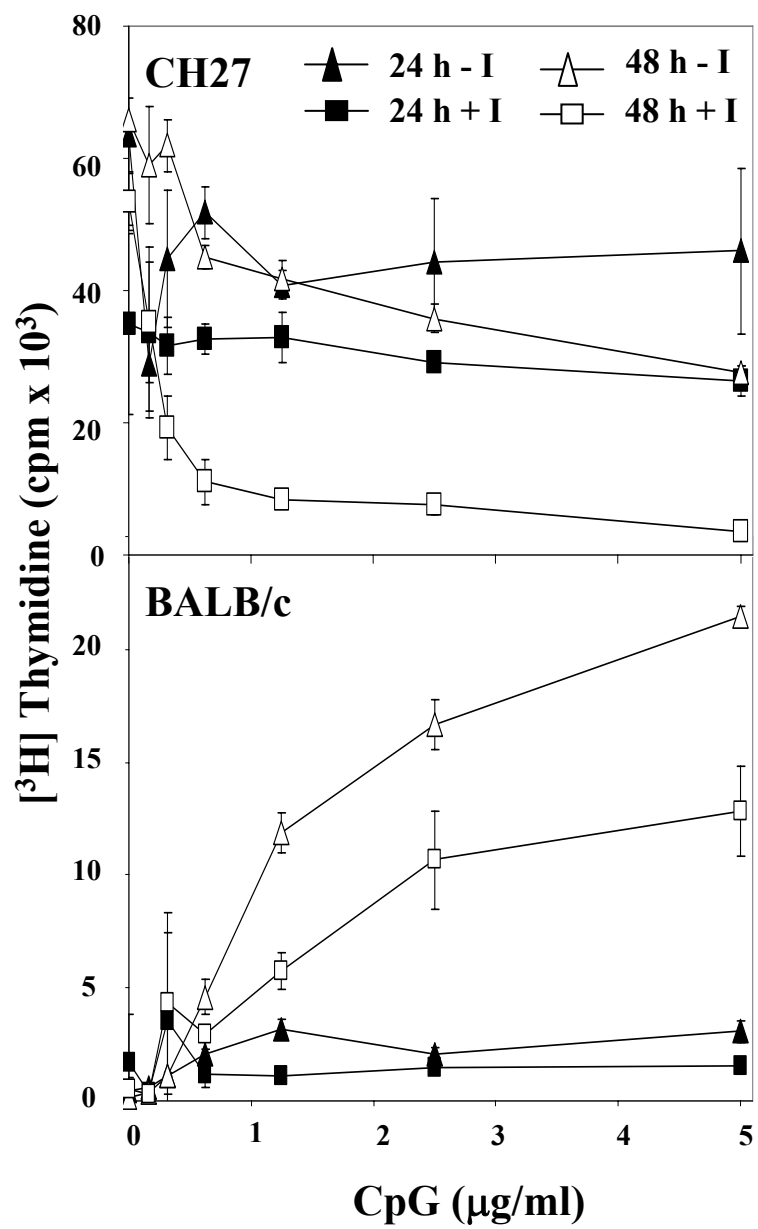


**Figure 2-6. Effect of NF- $\kappa$ B activators and inhibitor on proliferation of lymphoma and primary B cells.**

CH27 clone 1 cells were treated with varying concentrations of PMA or ionomycin or PMA and ionomycin (A) or CH27 clone 1 cells and splenic B cells with varying concentrations of NF- $\kappa$ B inhibitor in the presence or absence of 2.5  $\mu$ g/ml of GpC or CpG ODN (B) or CH27 clone 1 cells and splenic B cells were treated with varying concentrations of CpG or GpC DNA in the presence or absence of 5nM NF- $\kappa$ B inhibitor (C). The rate of proliferation of the cells was determined as described in Figure 2-1 except in (C) where the cells were harvested after 24 or 48 h of treatment. Data represent mean ( $\pm$  S.D.) of triplicate determinations. Shown are the representative results from three independent experiments.

**A****B**

C



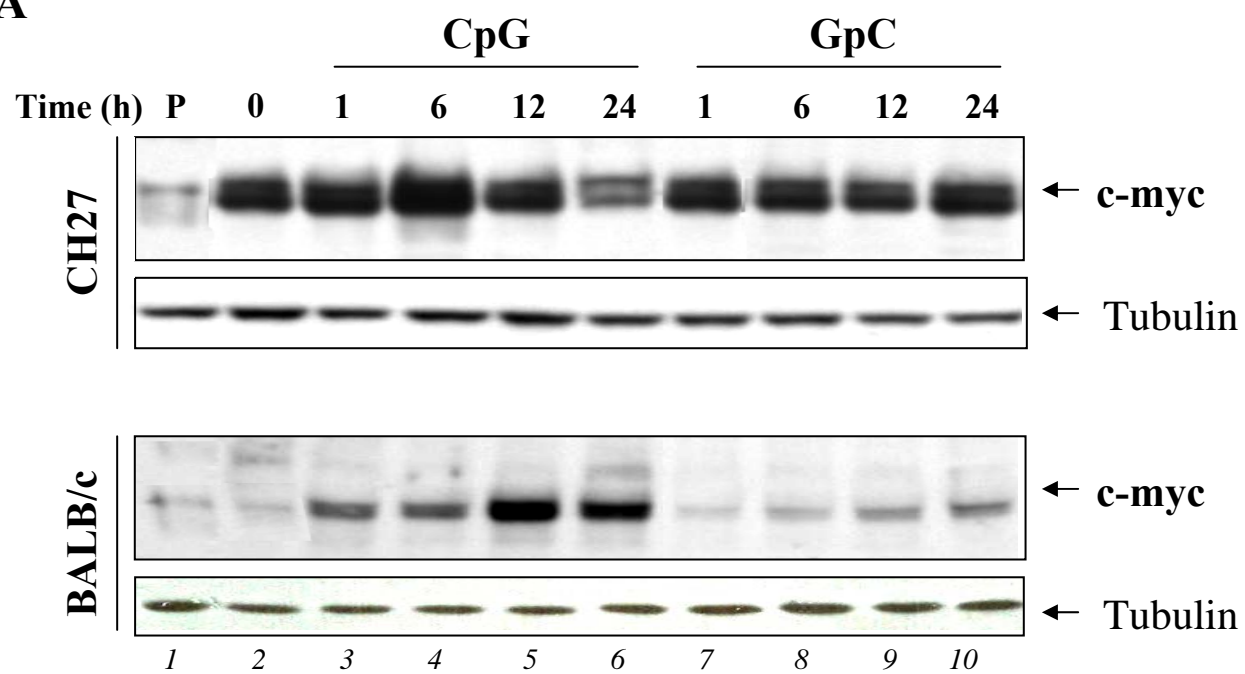
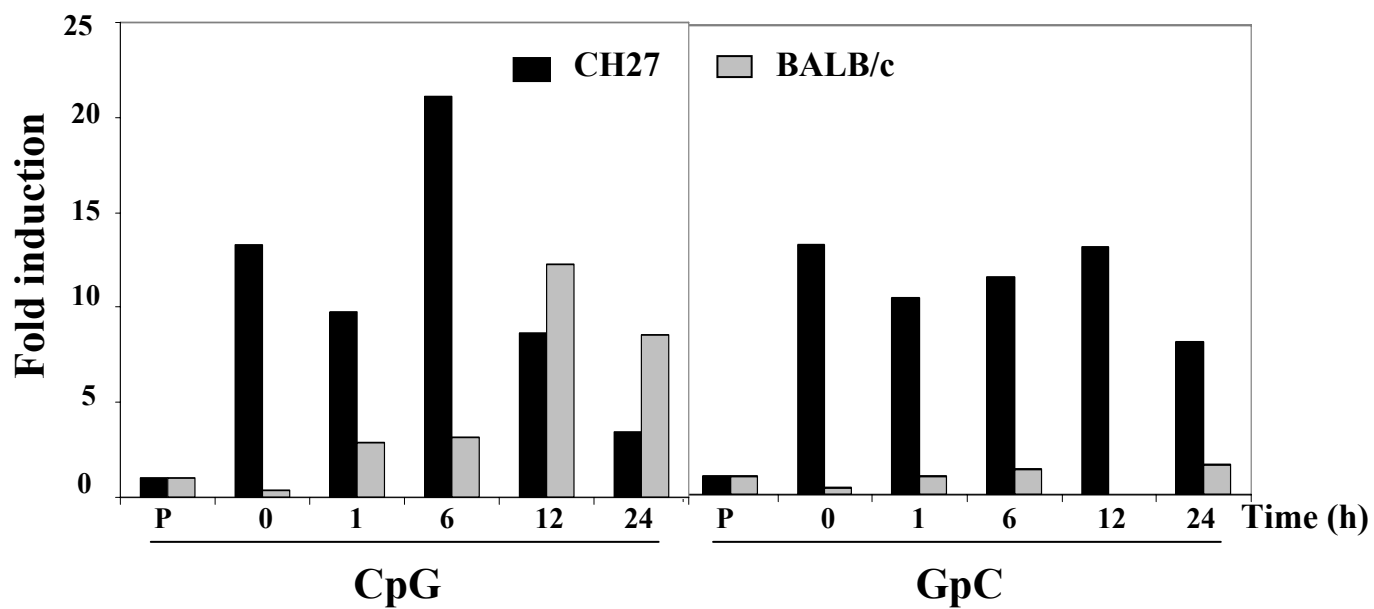
with NF- $\kappa$ B inhibitor (Fig. 2-6B). Furthermore, when CH27 and splenic B cells were treated with different concentrations of CpG or GpC ODN in the presence of NF- $\kappa$ B inhibitor similar reduction in rates of proliferation was observed (Fig. 2-6C). Hence, the NF $\kappa$ B inhibitor exacerbated the anti-proliferation effect of CpG ODN in the CH27 B cell lymphomas suggesting that CpG ODN-induced CH27 cell apoptosis is associated with the inhibition, but not the transient activation of NF- $\kappa$ B. This also indicates that NF- $\kappa$ B is crucial for CpG-induced proliferation in B cells.

#### **Differential effects of CpG ODN on the expression levels of c-myc in splenic and CH27 lymphoma B cells.**

c-myc is one of the key regulators in cell proliferation and survival. CpG DNA has been reported to induce an increase in c-myc protein levels, conferring a pro-survival and anti-apoptotic effect on splenic B cells [172]. On the other hand, one of the major causes of B cell lymphoma, such as Burkitt's lymphoma, is the aberrant translocation of the *c-myc* gene to the immunoglobulin heavy chain locus during recombination events, which leads to constitutive expression of c-myc [216]. To compare the effect of CpG DNA on c-myc expression in CH27 lymphomas and splenic B cells, cells were treated with 10  $\mu$ g/ml CpG ODN or control GpC ODN for 1, 6, 12 and 24 h, and the lysates were subjected to Western blot analyses to determine c-myc protein levels. As observed in many other B lymphomas [217], there was a high level of constitutively expressed c-myc protein in unstimulated CH27 lymphoma B cells (Fig. 2-7A, top panel, lane 2, and Fig. 2-7B), compared to the resting splenic B cells (Fig. 2-7A, bottom panel, lane 2, and Fig. 2-7B). Treating CH27 lymphoma B cells with CpG ODN increased c-myc protein levels at 6 h,

**Figure 2-7. Differential effects of CpG DNA on expression level of c-myc in lymphoma B and primary B cells.**

(A) CH27 lymphoma B cell clone 1 and splenic B cells were treated with or without 10  $\mu\text{g/ml}$  of GpC or CpG ODNs for specified times. Cells were then lysed and the lysates were subjected to SDS-PAGE and Western blotting, probed with anti-c-myc antibody and an HRP-conjugated secondary antibody. Shown is a representative blot of five independent experiments. **P** refers to a positive control of HeLa cell lysate. (B) The relative amount of c-myc protein expressed in CH27 lymphoma B cells clone 1 (black bars) and splenic B cells (grey bars) in (A) was quantified by densitometry and normalized to the relative amount of tubulin in the same sample. The results were plotted as the fold of densitometric value of the c-myc positive control sample, **P**.

**A****B**

followed by a significant decrease in c-myc protein at 12 h to a level less than the constitutive level at 24 h (Fig. 2-7A, top panel, lane 3 - 6, and Fig. 2-7B). However, CpG ODN treatment induced a sharp increase in c-myc expression in the splenic B cells, which became detectable at 1 h and continued to increase at 12 h after exposure to CpG ODN (Fig. 2-7A, bottom pane, lane 3 - 6, and Fig. 2-7B). Control GpC ODN had no significant effect on the expression of c-myc in both the cell types (Fig. 2-7A, lane 7 - 10, and Fig. 2-7B). Thus, CpG ODN treatment of CH27 lymphoma B cells that constitutively express a high level of c-myc, induced a transient increase, followed by a drastic decrease in c-myc protein levels, while inducing an increase in c-myc expression levels in primary splenic B cells.

#### **CpG ODN-induced apoptosis in lymphoma B cells is independent of MAP kinases.**

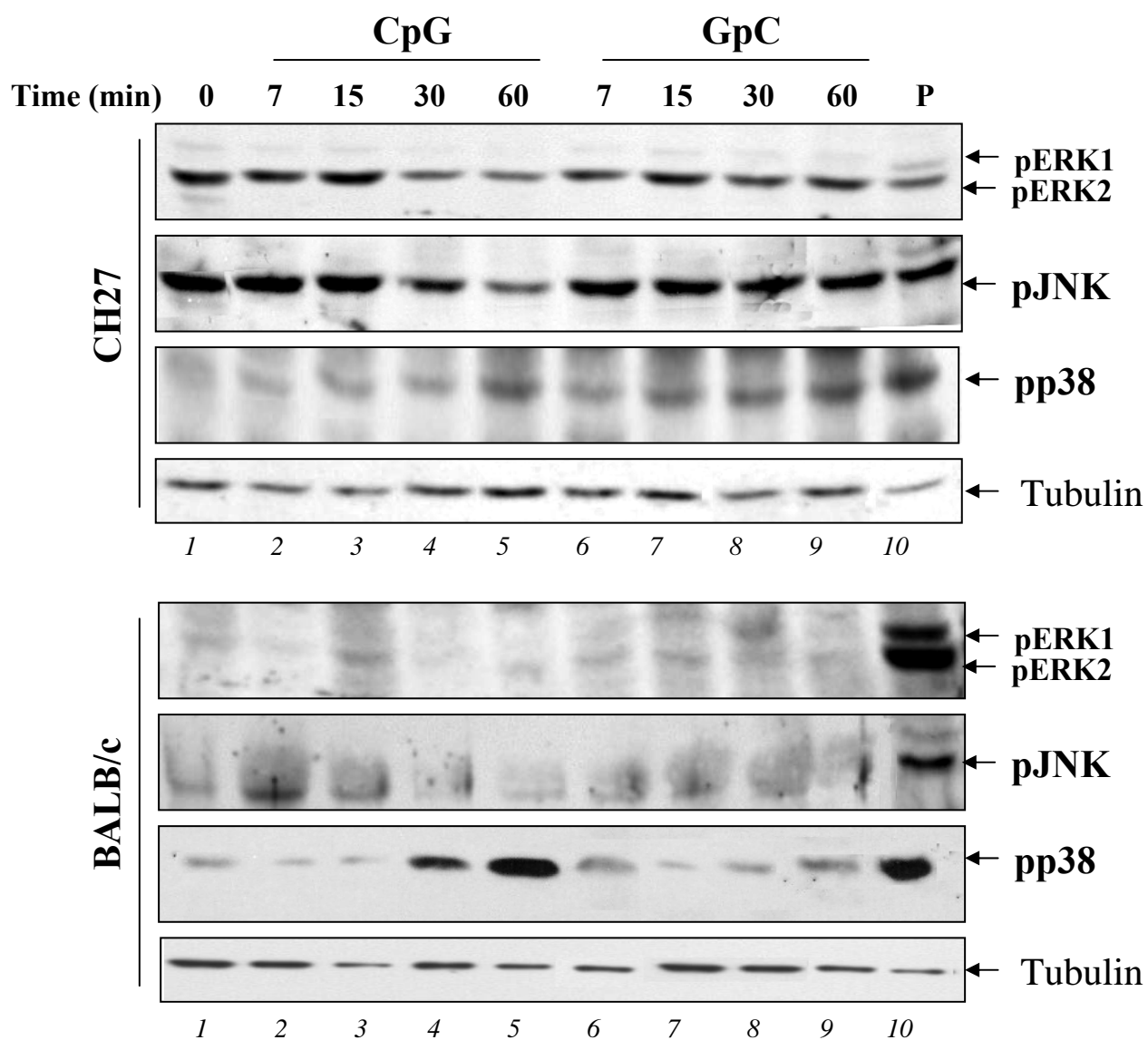
MAP kinases JNK and p38, which are activated by TLR9-induced signaling cascades in B cells, are important regulators of stressed-induced apoptosis [169, 170]. To test whether MAP kinases are involved in CpG ODN-induced apoptosis, we analyzed the effect of CpG ODN on the activation levels of ERK, JNK and p38 and the effect of MAP kinase inhibitors on the proliferation and apoptotic response of CH27 B cells to CpG DNA. The activation of the MAP kinases in CH27 and BALB/c splenic cells treated with CpG ODN was analyzed by Western blot analyses. CH27 lymphoma B cells had a constitutively high level of phosphorylated JNK (pJNK) and ERK (pERK) (Fig. 2-8, top panel, lane1). The levels of pJNK and pERK were significantly decreased after a 30 min treatment with CpG ODN and continued to decrease at 60 min (Fig. 2-8A, top panel,

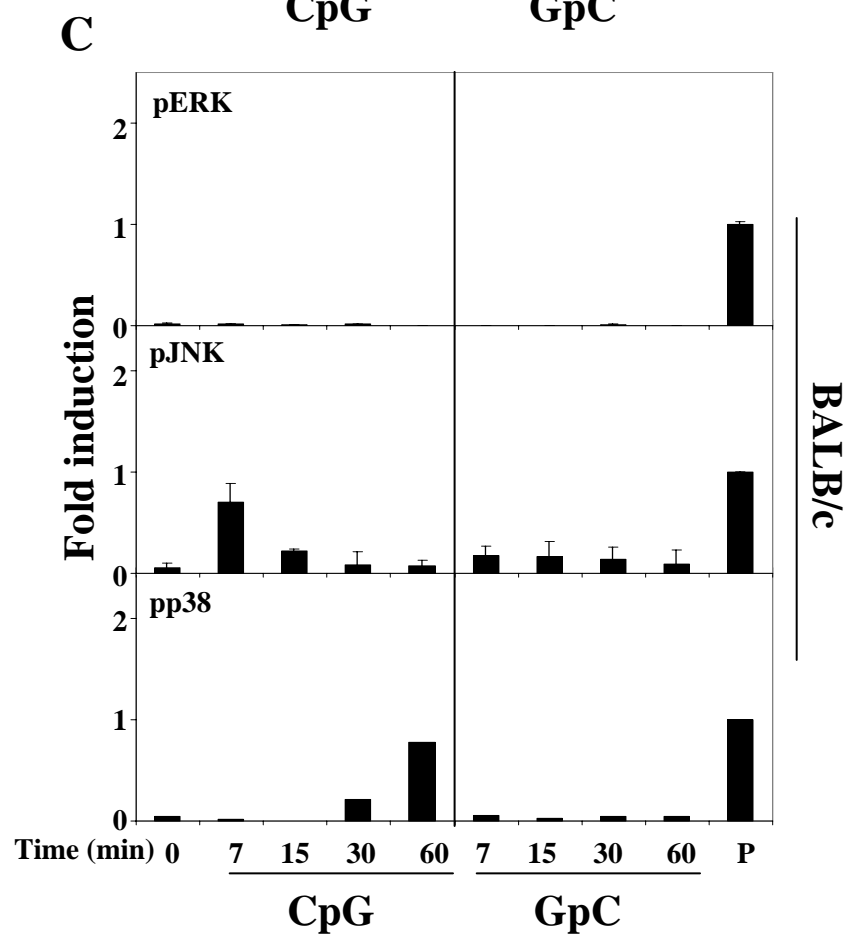
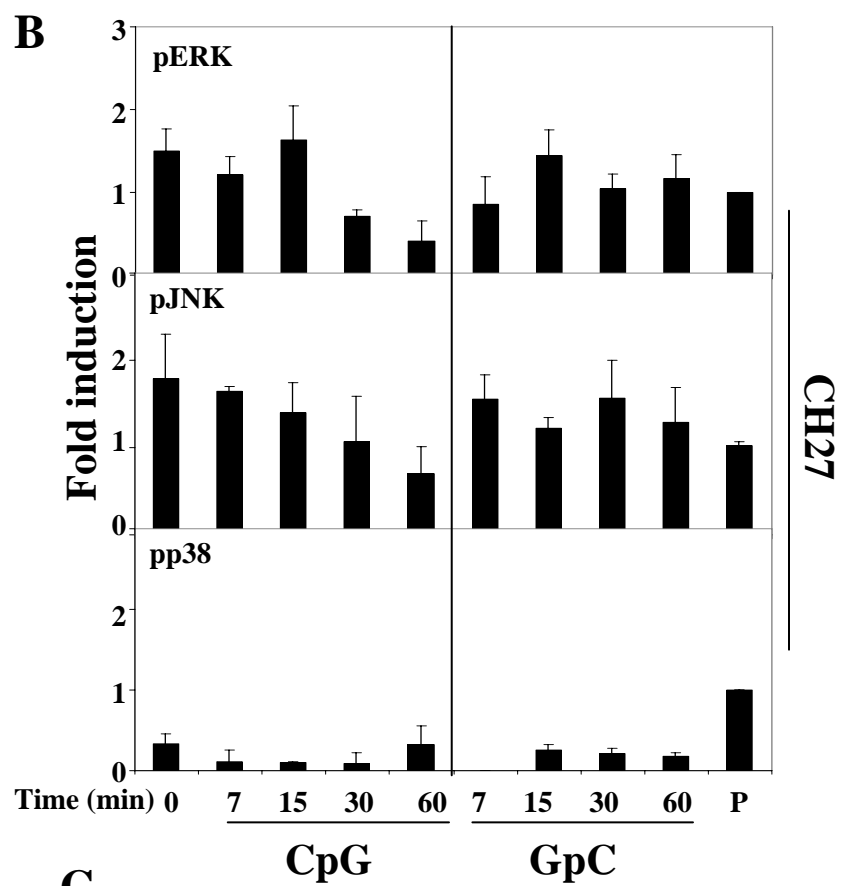
**Figure 2-8. CpG ODN-induced apoptosis in B cell lymphoma line is independent of MAP kinases.**

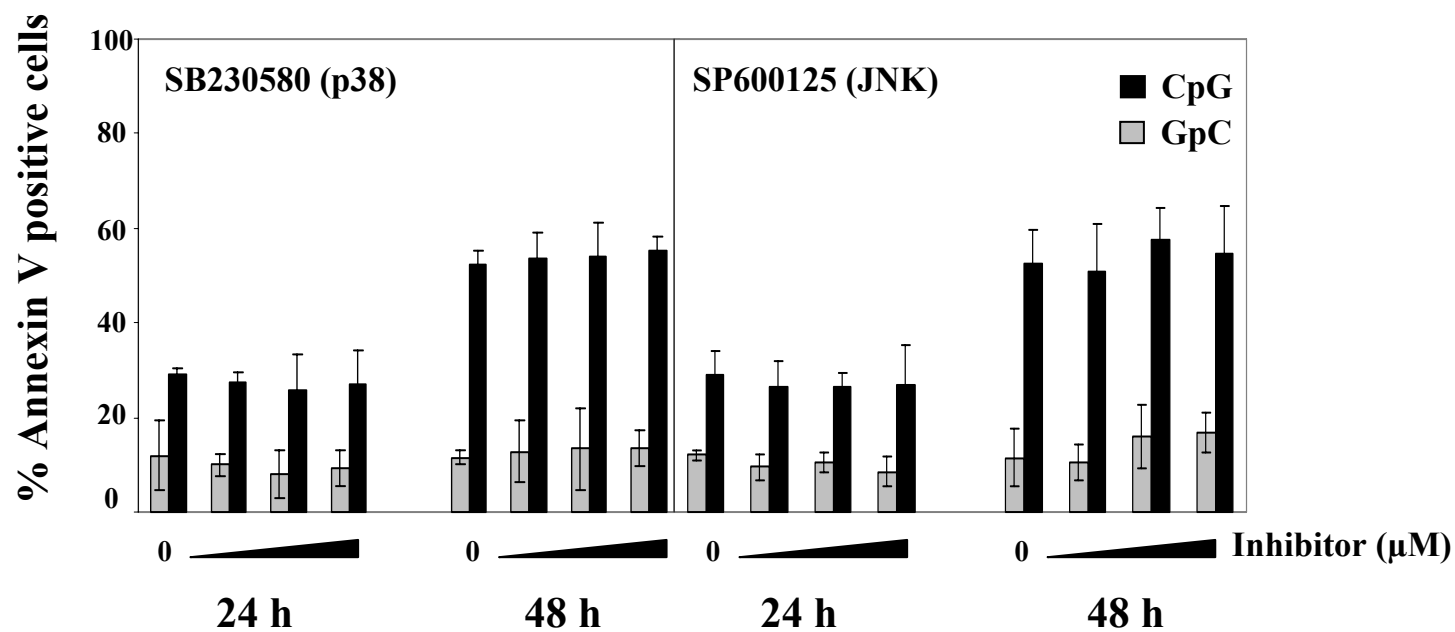
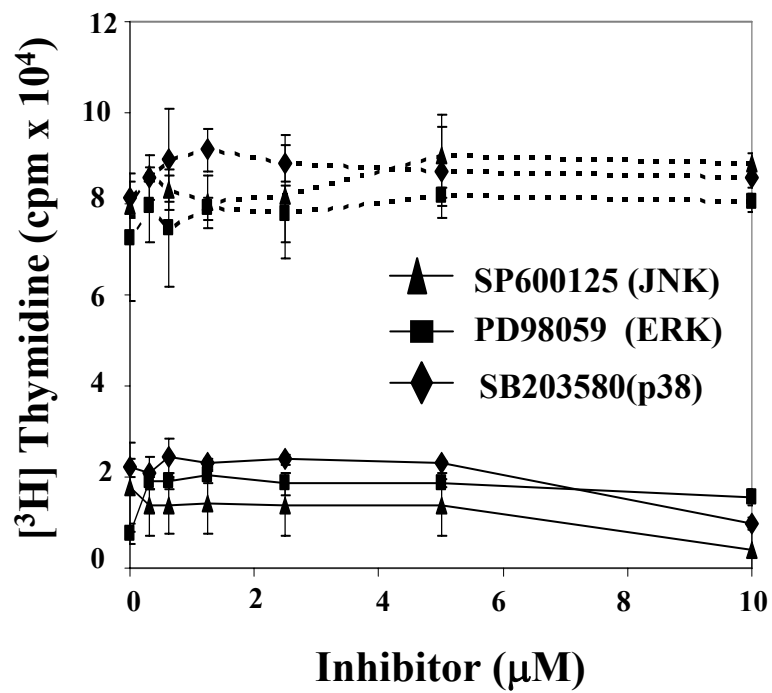
(A) CH27 B cell lymphomas clone 1 and splenic B cells were treated with or without 10  $\mu\text{g/ml}$  of GpC and CpG ODNs for specified times. Cells were then lysed and the lysates were subjected to SDS-PAGE and Western blotting. The blots were probed with antibodies specific for the phosphorylated forms of ERK or JNK or p38 and HRP-conjugated secondary antibodies. Shown are representative blots of five experiments. Lysates of splenic B cells that were stimulated by cross-linking the surface BCR with anti-Ig antibodies were used as positive control, **P**. (B and C) The relative amount of phosphorylated MAP kinase protein expressed in CH27 lymphoma B cells clone 1 (B) and splenic B cells (C) in (A) was quantified by densitometry and normalized to the relative amount of tubulin in the same sample. The results were plotted as the fold of densitometric value of the positive control, **P**. (D) CH27 B cell lymphomas clone 1 was incubated with increasing concentrations of JNK or p38 inhibitors (1, 5 and 10  $\mu\text{M}$ ) in the presence of CpG or GpC DNA (10  $\mu\text{g/ml}$ ). Rate of apoptosis induction was determined as described in Figure 2-3. Shown are the percentages ( $\pm$  S.D.) of Annexin V positive apoptotic cells from three independent experiments. (E) CH27 B cell lymphomas clone 1 was incubated with increasing concentrations of inhibitors specific for JNK, ERK, and p38 in the presence of GpC (dotted lines) or CpG (straight lines) ODN (10  $\mu\text{g/ml}$ ). Cell proliferation was determined as described in Figure 2-1. Data represent mean ( $\pm$ S.D.) of triplicate samples. Shown are the representative results from three independent experiments.



**A**





**D****E**

lane 2 - 5 and Fig. 2-8B). Phosphorylated JNK was undetectable in untreated splenic B cells and briefly detected at 7 min after CpG ODN treatment (Fig. 2-8A, bottom panel lane 2 and Fig. 2-8B), while phosphorylated ERK was undetectable at all time points tested (Fig. 2-8A, bottom panel, lane 1 - 9 and Fig. 2-8C). CH27 lymphoma B cells and splenic B cells have constitutively low level of phosphorylated p38 (Fig. 2-8A, top and bottom panel, lane 1, Fig. 2-8B and Fig. 2-8C). The p38 levels in CH27 lymphoma B cells do not change upon treatment with CpG ODN (Fig. 2-8A, top panel, lane 1 - 9 and Fig. 2-8B), while it appears to be upregulated in splenic B cells at 30 min of treatment (Fig. 2-8A, bottom panel, lane 4 and Fig. 2-8B).

To inhibit MAP kinase activation, CH27 cells were treated with varying concentrations of inhibitors of JNK (SP600125) and p38 (SB203580) [170] in the presence or absence of 10 µg/ml CpG or GpC ODN. Compared to cells treated with CpG ODN alone, addition of the inhibitors did not significantly change the proliferation rate (Fig. 2-8E) or apoptosis level (Fig. 2-8D) of CH27 cells. Furthermore, treating CH27 cells with inhibitor specific for ERK activation (PD98059) in the presence or absence of CpG or GpC ODN did not affect the proliferation rate of CH27 cells (Fig. 2-8E). This suggests that CpG ODN-induced apoptosis in CH27 lymphoma B cells is independent of MAP kinases JNK, ERK, and p38.

#### **Effect of LPS on CH27 B cell lymphomas.**

Lipopolysaccharide (LPS) found on the cell walls of Gram negative bacteria is a potent stimulus for B cell proliferation [40]. LPS binds Toll-like receptor 4 (TLR4) and activates

NF- $\kappa$ B through a MyD88-dependent signaling pathway [218]. We tested the effect of LPS on the CH27 lymphoma B cells. CH27 B lymphoma cells were cultured in the absence of or with varying concentrations of LPS for 48 h, and the rate of cell proliferation was detected using [ $^3$ H]-thymidine incorporation. Similar to treatment with CpG ODN, increasing concentrations of LPS induced a sharp decrease in the proliferation of the lymphomas, compared to untreated cells (Fig. 2-9A). LPS induces NF- $\kappa$ B activation in B cells similar to CpG ODN. To follow the activation of NF- $\kappa$ B, the degradation of the inhibitory component of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , in CH27 lymphomas treated with LPS was followed by Western blot. LPS induced a transient decrease of I $\kappa$ B $\alpha$  level at 30 min, but returned to basal level by 120 min in the CH27 lymphomas (Fig. 2-9B), while the untreated samples did not alter the protein levels of I $\kappa$ B $\alpha$  (Fig. 2-9B).

Taken together these data suggest that like CpG ODN, LPS inhibits the proliferation of CH27 lymphomas and causes transient NF- $\kappa$ B activation.

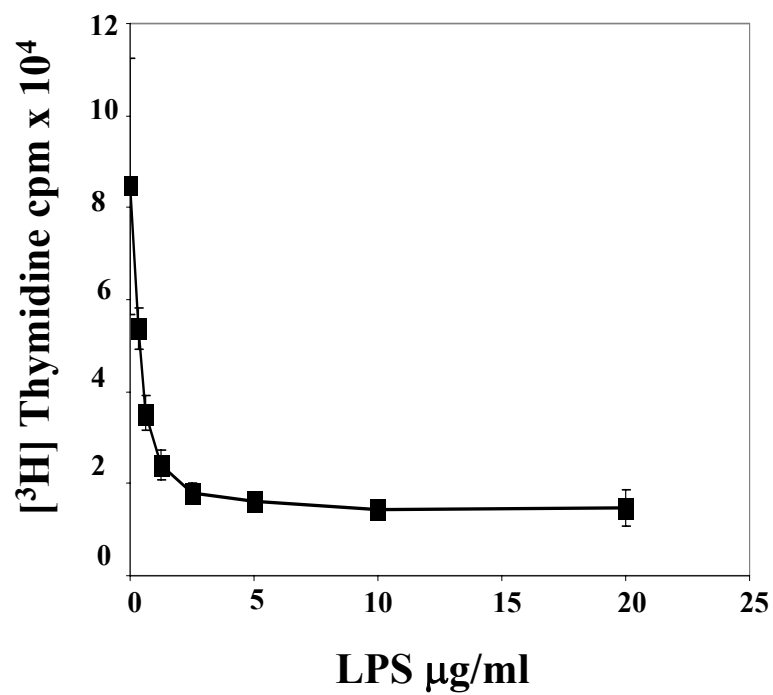
### **B cell lymphoma cells internalize and transport CpG DNA to late endosomes.**

The different cellular responses of B lymphomas and splenic B cells to CpG ODN might occur due to different or impaired internalization of CpG ODN in the CH27 B lymphomas. To test this, we followed the internalization of biotinylated CpG by immunofluorescence microscopy. Surface IgM, which is known to rapidly internalize and move to late endosome upon cross-linking [219], was stained as a control in both cell types. We observed internalization of the CpG ODN by 30 min of exposure in both the

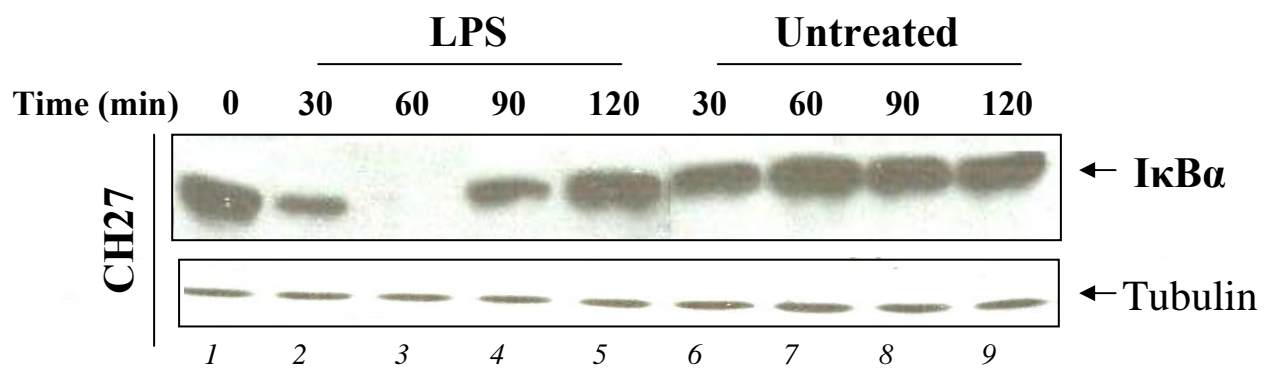
**Figure 2-9. Effect of LPS on CH27 B cell lymphomas**

(A) CH27 B cell lymphomas clone 1 was incubated with increasing concentration of LPS. Cell proliferation was determined as described in Figure 2-1. Data represent mean ( $\pm$ S.D.) of triplicate samples. Shown are the representative results from three independent experiments. (B) CH27 lymphomas clone 1 ( $2 \times 10^6$  cells/ml) was treated in the presence or absence of  $1\mu\text{g/ml}$  LPS for various times (30 min to 2 h). Lysates were prepared and probed with anti- $\text{I}\kappa\text{B}\alpha$  antibody as described in Figure 2-5C.

**A**



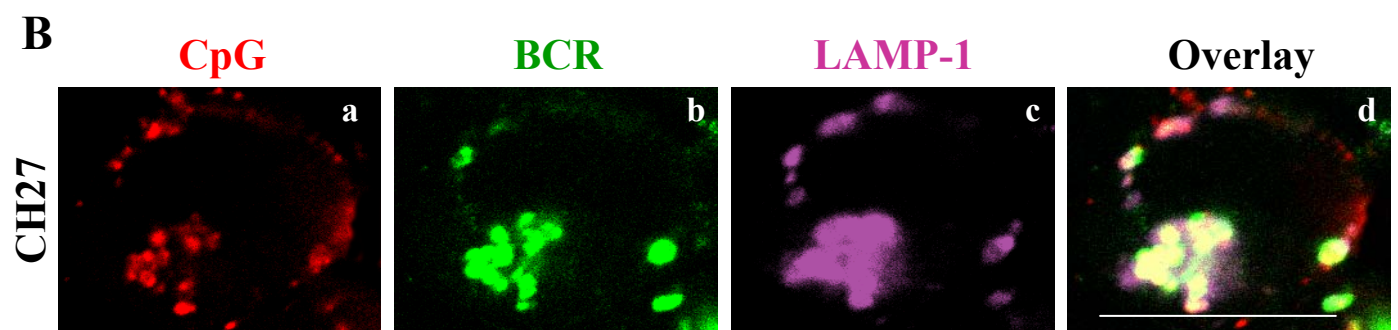
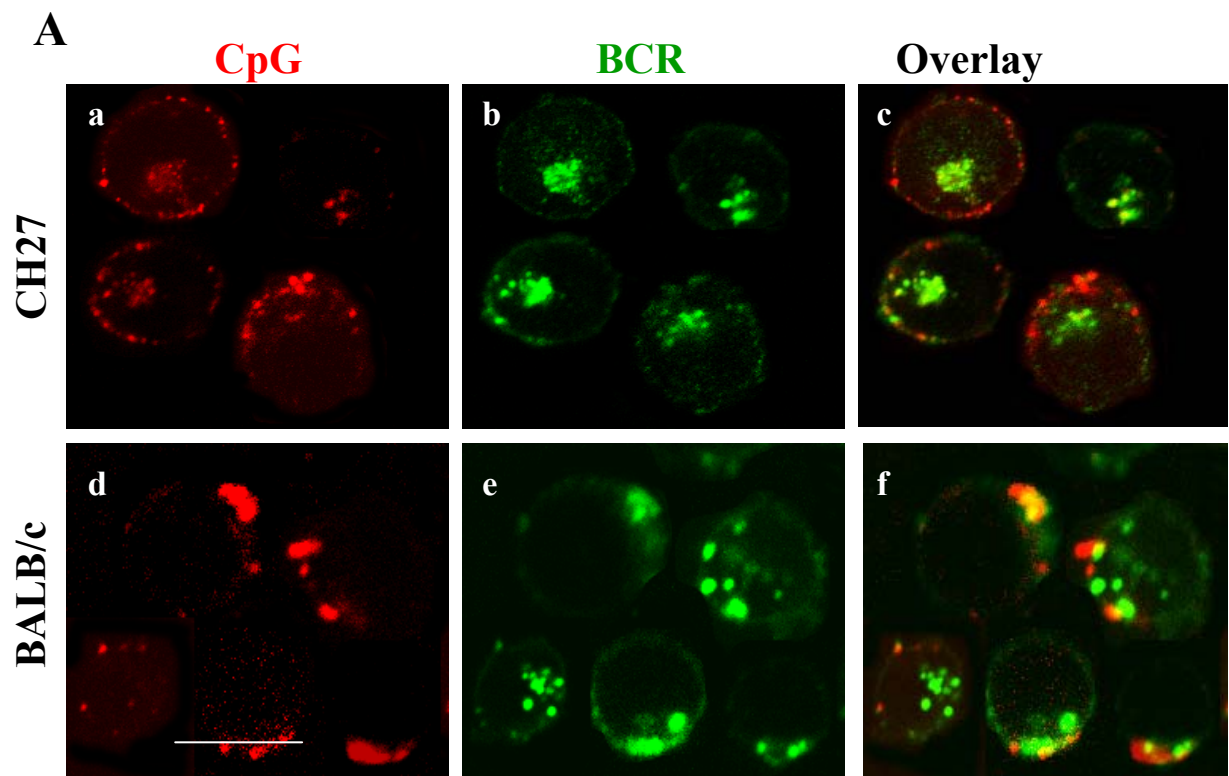
**B**



**Figure 2-10. B cell lymphoma cells internalize and transport CpG DNA to late endosomes.**

CH27 lymphomas (Aa – Ac) and splenic B cells (Ad – Af) were treated with 7µg/ml biotinylated CpG and FITC goat anti-IgM for various times at 37°C. The cells were then fixed, permeabilized and incubated with AlexaFluor 546- streptavidin alone (A) and anti-mouse LAMP-1 mAb (ID4B) and goat anti-mouse IgG secondary antibody (B). Images were acquired in the middle of the cells using a confocal fluorescence microscope. Shown are representative images of cells after 30 min of exposure to ODN. Bar, 10 µm





CH27 B cell lymphomas (Fig.2-10Aa – Ac) and splenic B cells (Fig.2-10Ad - Af), indicating no significant difference in the internalization of the ODNs between primary splenic B cells and B lymphoma cells. Dendritic cells and macrophages have been shown to internalize CpG ODN and traffic them to LAMP-1 positive late endosome/lysosomes compartments [119, 136]. To determine the cellular distribution of internalized CpG ODN in the CH27 B cell lymphomas, the late endosomal/lysosomal compartments were visualized with anti-LAMP-1 mAb. At 30 min, CpG ODN co-localizes with LAMP-1 positive late endosomal/lysosomal compartments (Fig 2-10Bd), indicating that internalized CpG ODN moved to the endosomal/lysosomal vesicles in CH27 B cell lymphomas

## 2-5 Discussion

The recognition of common molecules present on microbial pathogens by Toll-like receptors initiates innate immune responses. Recent studies showed that the binding of ligands to TLR2, 3, 4, 6 and 9 leads to the apoptosis of cancer cells and activated macrophages [188, 189, 191, 196]. While TLR-induced cell death has been suggested to be a negative feedback mechanism for preventing over-activated inflammatory responses [203], its underlying mechanisms and physiological significance remain to be elucidated. This study examined the differential effects of TLR9 ligand, CpG ODN, on naïve mouse splenic B cells and mouse lymphoma B cells and explored the underlying mechanism for the pro-apoptotic function of TLR9. It was found that while it promoted the proliferation of splenic B cells, TLR9 ligand, CpG ODN, induced apoptosis in CH27 lymphoma B cells. CpG ODN-induced apoptosis was associated with a transient activation of NF- $\kappa$ B, an increase of c-myc over an already high level of constitutively expressed c-myc, and decreases in the levels of anti-apoptotic molecule Bcl-xl and phosphorylated MAP kinase JNK that were constitutively high in CH27 cells.

Several recent publications have reported TLR9-triggered cell death. Fischer *et al.* showed that TLR9 ligand sensitized a TLR9-transfected fibroblast cell line for apoptosis [195]. Jahrsdorfer *et al.* recently reported that cells from biopsy samples of patients suffering from B cell chronic lymphocytic leukemia (B-CLL) underwent apoptosis when treated with CpG ODN [196]. Similar to these reports, we found in this study that CpG ODN induced apoptosis of a mouse B cell lymphoma line, CH27. This further supports a pro-apoptotic effect of TLR9 ligands on tumor cells that express TLR9.

The CH27 murine B cell lymphoma was generated from the peritoneal cavity of 2<sup>a4b</sup> mice that were repeatedly immunized with chicken red blood cells [197]. CH27 cells predominantly express mIgM that is specific for phosphorylcholine [198], suggesting that it originated from B1 lineage. Here we showed that CH27 cells constitutively express high levels of Bcl-xl and c-myc, two genes that are commonly deregulated in cancer cells and translocated to Ig H locus in cancer B cells, such as human Burkitt's lymphoma [220]. The CH series of lymphomas was also thought to exhibit phenotypic characteristics similar to human B cell chronic lymphocytic leukemia (B-CLL) [198]. It has been suggested that B-CLLs arise due to chronic antigenic stimulation, most probably from autoantigens [221]. Surface immunoglobulins of B-CLLs react with self-antigens like DNA and Ig [222]. There has been at least one case of B-CLL expressing IgM specific for phosphatidylcholine [223]. Hence the CH27 B lymphomas can serve as an *in vitro* model to understand the underlying mechanism for the pro-apoptotic functions of TLR9 on B cell cancers.

Our results showed that only CpG ODN that contained the CpG motif, but not GpC ODN that had the CpG motif inverted, exhibited immunostimulatory and pro-apoptotic effect. While GpC ODN has served as an ideal negative control for our experiments, the results from these experiments also allude to the sequence specific nature of these effects. In addition, we found that CpG ODN only induced apoptosis in CH27 B cell lymphoma cells that express TLR9 mRNA, but had no effect on the cell proliferation and apoptosis of a variant of the CH27 cells that does not express TLR9. This demonstrates that CpG ODN-induced apoptosis in CH27 lymphoma B cells is mediated by TLR9. Interestingly,

CH27 lymphoma B cells that were treated with lipopolysaccharide (LPS) which activates TLR4, also showed a similar reduction in rate of proliferation. TLR4 activates MyD88 pathway, similar to CpG ODN. This suggests that both CpG ODN and LPS utilize a common TLR pathway to induce their anti-proliferative effects.

To further understand the mechanism for CpG ODN-induced apoptosis of CH27 lymphoma B cells, we compared the effect of CpG ODN treatment on the levels of cleaved caspase 3, an executioner caspase, and two major Bcl-2 family proteins, Bcl-xl and Bax that regulate the mitochondrial stability, in the two types of cells. Bcl-xl is an anti-apoptotic and Bax a pro-apoptotic protein. Our data showed that CpG ODN induced the cleavage of caspase 3 in CH27 lymphoma B cells, but not in splenic B cells, suggesting that CpG ODN-induced apoptosis in CH27 cells is mediated by a caspase-dependent pathway. In untreated CH27 cells, we detected a significant level of Bcl-xl, and the constitutive expression of Bcl-xl is likely to be part of tumorigenic mechanisms in CH27 cells. Instead of increasing Bcl-xl protein levels as seen in naïve primary B cells, CpG ODN treatment drastically decreased Bcl-xl protein levels in CH27 lymphomas. Because Bcl-xl is essential for protecting the integrity and stability of mitochondria and inhibiting mitochondria-mediated apoptosis pathway, a drastic decrease in Bcl-xl could be a direct course for the apoptosis of the CH27 B cell lymphomas by destabilization of mitochondria. CpG ODN treatment had no significant effect on Bax protein levels in CH27 cells. Unaltered Bax level does not exclude it from playing a role in CpG ODN-induced apoptosis since the activation of Bax during apoptosis generally does not require an increase in its expression level [224]. The Bcl-2 family comprises many members,

including Bax, Bak, Bok, Bid, Bad, Bim, Puma and Noxa [225]. In addition to Bcl-xl and Bax, the other members of the Bcl-2 family could also play roles in CpG ODN-induced apoptosis of CH27 lymphomas.

NF- $\kappa$ B and c-myc have emerged as two key transcription factors that regulate the balance between cell proliferation and apoptosis [226, 227]. NF- $\kappa$ B is crucial for host defense [152] and essential for B cell survival during B cell development and differentiation [153]. Besides activating genes encoding for cytokines and immune receptors, NF- $\kappa$ B induces the transcription of cell cycle regulators, like cyclin D1 [158] and c-myc [159], and anti-apoptotic genes like Bcl-xl [161, 162], which promote cell cycle progression and cell survival. The NF- $\kappa$ B family comprises multiple members, and Rel-A/p65 is a major member of the NF- $\kappa$ B family expressed in B cells. Rel-A/p65-deficient mice have greatly diminished numbers of B cells and are sensitive to TNF-mediated apoptosis [153]. c-myc induces the expression of cell cycle proteins and is essential for the progression of G1 stage of the cell cycle [228]. NF- $\kappa$ B or c-myc has been found to be constitutively activated or expressed in a number of human B cell lymphomas and have been implicated for tumorigenesis [229]. Paradoxically, inappropriate activation of Myc and NF- $\kappa$ B can also promote or sensitize cells to apoptosis [230]. Myc has long been known as an inducer of apoptosis [230, 231]. Recent studies have provided strong evidence that cell proliferation induced by Myc in the absence of sufficient levels of anti-apoptotic signals leads to apoptosis (see review [232, 233]). NF- $\kappa$ B has been shown to induce apoptosis in certain types of cells stimulated under specific conditions (see review [161]). Both NF- $\kappa$ B (see review [161]) and c-myc (see review [232]) can induce apoptosis by directly or

indirectly regulating the expression levels of apoptotic regulators like Bcl-xl, cell cycle checkpoint protein like p53, and death receptors like CD95. Cooperation between NF- $\kappa$ B and c-myc is essential for controlling cell fate. c-myc induced cell death was accompanied by impaired activation of NF- $\kappa$ B and release of cytochrome c from the mitochondria [234] and over expression of the p65 subunit of NF- $\kappa$ B rescued cells from c-myc dependent cell death [235].

In this study, we found that CpG ODN-induced apoptosis in CH27 B cell lymphoma was concurrent with a transient increase and a subsequent rapid decrease in NF- $\kappa$ B activity and c-myc protein level, which is in sharp contrast with naïve splenic B cells where CpG ODN induced a steady increase in NF- $\kappa$ B activity and c-myc protein level. Another important difference that we observed between CH27 cells and splenic B cells is that CH27 cells, but not splenic B cells, constitutively express a significant level of c-myc. CpG ODN treatment further increased the c-myc protein level around 6 h. Differing from a previous report [236], we did not observe constitutive activation of NF- $\kappa$ B in CH27 cells. After transient activation by CpG ODN, NF- $\kappa$ B activation level rapidly decreased; returning to the basal level at 90 min. LPS treatment also induced a similar transient activation of NF- $\kappa$ B in the CH27 lymphoma B cells. Following the decrease in NF- $\kappa$ B activity, c-myc protein level also decreased to the constitutive level at 12 h and to a level lower than the constitutive level by 24 h in the lymphoma cells treated with CpG ODN. Interestingly, a similar correlation between the activity level of NF- $\kappa$ B and c-myc expression level during B cell antigen receptor-triggered apoptosis in an immature B cell lymphoma line, WEHI 231, were reported [237, 238]. These observations point to the

deregulation of NF- $\kappa$ B and c-myc as a mechanistic cause of CpG ODN-induced apoptosis in CH27 cells. LPS-induced transient NF- $\kappa$ B activation in CH27 lymphoma cells, suggests that the reduction in proliferation of CH27 cells might occur by a similar deregulation of NF- $\kappa$ B activation. Based on previous studies and the data presented here, we propose several possible mechanisms for CpG ODN-induced apoptosis. First, CpG ODN-induced NF- $\kappa$ B activation may trigger apoptosis, secondly CpG ODN-induced aborted activation of NF- $\kappa$ B causes the down-regulation of anti-apoptotic genes, leading to apoptosis, and thirdly CpG-ODN-induced further elevation of c-myc protein over its constitutively high level may cause apoptosis.

To test whether CpG ODN-induced CH27 apoptosis is due to the initial hyper-activation or the later hypo-activation of NF- $\kappa$ B, we tested the effect of activating NF- $\kappa$ B through protein kinase C and inhibiting NF- $\kappa$ B activation on CH27 cell proliferation. We found that activating protein kinase C slightly increased CH27 cell proliferation, and NF- $\kappa$ B inhibitor further enhanced the inhibitory effect of CpG ODN on CH27 cell proliferation and decreased CpG-induced proliferation of splenic B cells. This indicates that CpG ODN-induced apoptosis of CH27 cells is, at least in part, the result of hypo-activation of NF- $\kappa$ B. Our data showed that while NF- $\kappa$ B activity was rapidly reduced, c-myc protein level remained high. Imbalance between the decreasing activity of NF- $\kappa$ B and a constitutive high level of c-myc could generate a situation, where cell proliferation induced by c-myc in the absence of sufficient anti-apoptotic signals from NF- $\kappa$ B, culminates in apoptosis.



TLR9-mediated signaling pathway activates MAP-kinase JNK and p38 in B cells [169, 170], which are also important apoptotic regulator in B cells. Stress-induced activation of JNK and p38 often leads to apoptosis through activation of mitochondrial apoptotic pathway [239, 240]. NF- $\kappa$ B activation suppresses JNK activation, which protects cells from apoptosis [241] and p38 suppression by use of pharmacological inhibitors leads to apoptosis [242]. However, apoptosis induced by over-expression of c-myc has been reported to associate with a decrease in JNK phosphorylation, and c-myc can inhibit upstream kinases of MAP kinases, like MEKK1, impairing both the NF- $\kappa$ B and JNK activation [235]. Paradoxically, many human and mouse B cell lymphomas constitutively express high levels of active JNK, and the survival and proliferation of these B cell lymphomas depend on JNK activation [243]. In this study, we found that JNK was constitutively activated in CH27 cells, similar to other lymphoma B cells. CpG ODN treatment significantly reduced the level of phosphorylated JNK but did not activate p38 in the CH27 lymphomas. Post-translational stability of c-myc is controlled by phosphorylation of specific serine or threonine residues of c-myc protein. ERK increases stability of c-myc protein by phosphorylating serine 62 [244]. We found that ERK was constitutively activated in CH27 lymphomas and CpG ODN treatment decreased the level of phosphorylated ERK. This suggests that in addition to NF $\kappa$ B, the decrease in c-myc protein levels might also be regulated by decreasing levels of activated ERK in CH27 B cell lymphomas treated with CpG ODN. However, treating CH27 cells with inhibitors specific to MAP kinase JNK, ERK or p38 had no significant effect on the constitutive proliferation of CH27 cells, and neither restored nor enhanced the inhibitory effect of CpG ODN on CH27 cell proliferation. This suggests that MAP-kinases may not

be essential for constitutive proliferation and CpG ODN-induced apoptosis of CH27 lymphoma B cells.

This study, using a mouse lymphoma B cell line as a model, demonstrated that CpG ODN induces apoptosis in lymphoma B cells that constitutively express c-myc and Bcl-xl in a TLR9-dependent manner. Our study further reveals that TLR9-induced abortive activation of NF- $\kappa$ B accompanied by an over-elevated c-myc level is a likely underlying mechanism for CpG ODN-induced apoptosis. Since several common human lymphomas, such as B-CLL and Burkitt's lymphoma, have properties similar to CH27 lymphoma B cells, it will be extremely interesting to test whether CpG ODN can induce apoptosis in these lymphomas by a similar mechanism. Further studies are required to fully understand the mechanism for TLR-induced apoptosis.

TLR9 agonists strongly trigger both innate and adaptive immune responses, making them desirable for use as vaccine adjuvants, in cancer and allergy therapy. TLR9 agonists are in clinical trials as vaccine adjuvants for cancer and infectious diseases. CpG 7909 is in final phase of clinical trials for treatment of non small cell lung cancer. In these clinical trials patients undergoing a combination treatment of CpG ODN and monoclonal antibody therapy or chemotherapy were shown to have better rates of tumor regression and survival. Our data reported here, further supports the use of TLR9 agonists to enhance targeted deletion of cancer cells by activating the host immune system.

## **Chapter 3: Polyvalent Antigens Stabilize B Cell Antigen Receptor Signaling Microdomains**

### **3.1 Abstract**

The B cell antigen receptor (BCR) can distinguish subtle differences in antigen structure and trigger differential responses. Here, we analyzed the effects of antigen valency on the functions of the BCR using three different antigen systems – anti-BCR antibody – based antigens, phosphorylcholine (PC)-based antigens and hen egg lysozyme (HEL)-based antigens. While both paucivalent and polyvalent antigens induced the redistribution of surface BCR into microdomains, polyvalent antigen-induced BCR surface microdomains persisted. Significantly, this trend was consistently observed in all three antigen systems studied. Ganglioside G<sub>M1</sub> and tyrosine-phosphorylated proteins and phosphorylated ERK co-localized with BCR microdomains, suggesting these function as surface signaling microdomains. Co-receptor, CD19 and MHC class II molecules accumulated in the BCR surface microdomains, but CD45 and the transferrin receptor were not preferentially concentrated in the microdomains. The cellular distribution of microtubule filaments was also influenced by antigen valency. Prolonged BCR caps were also concomitant with a reduction in BCR movement to late endosomes/lysosomes. Thus, antigen valency influences B cell responses by modulating the stability of BCR-signaling microdomains and BCR-mediated antigen transport.

### 3.2 Introduction

B cells express clone-specific receptors, the B cell antigen receptors (BCR), which sense the presence of antigens. B cell responses are initiated by the engagement of antigens to the BCR. The binding of antigens to the BCR initiates signaling cascades, leading to the expression of genes involved in cell proliferation and differentiation [245, 246], and induces the internalization of antigens for processing and presentation, acquiring T cell help [20, 247].

Unlike the T cell antigen receptor, the BCR recognizes antigens in their native forms with varying affinity, valency, chemical component and stereochemical structure. BCR engagement can initiate cellular events leading to a wide range of responses, including anergy, apoptosis, proliferation, differentiation, or memory B cell generation. The outcome of BCR engagement is influenced by the properties of antigens, differentiation stage of B cells, availability of T cell help, and environment of B cells. In the absence of T cell help, a productive B cell response requires thresholds of antigen concentration, affinity, and valency [40, 48, 248]. Below these thresholds, MHC class II-restricted T cell help is required. The relationship between antigen affinity and antigen-triggered responses has been extensively studied. Increasing antigen-BCR affinity has been shown to enhance B cell antigen presenting ability, T cell-independent B cell proliferation, antibody secretion, and interleukin 2 and interferon- $\gamma$  secretion *in vitro* [75, 76]. Using transgenic mice carrying antibody genes with a high or low antigen binding affinity, Shih et al. [72, 74] showed that while high- and low-affinity B cells had the same intrinsic capacity to respond to T-dependent antigens, high-affinity B cells generated a two-fold

higher response to T cell-independent antigens than low-affinity B cells. When both high- and low-affinity B cells were co-transferred to recipient mice, only high-affinity B cells responded to both T cell-dependent and independent antigens [72] and high affinity germinal center B cells are actively selected to differentiate into plasma cells [73].

The effect of antigen valency on B cell responses has also been examined. Increasing antigen valency by conjugating BCR-specific antibodies to dextran, Ficoll, Sepharose, or polyacrylamide beads reduced the amount of antigens required for induction of B cell proliferation [77, 78]. Conversely, increasing antigen valency using biotinylated anti-BCR antibodies and avidin or by coating anti-BCR antibodies to plastic or erythrocyte surface induced abortive activation of B cells and apoptosis [80-82]. Recently, Kim et al., reported that oligomeric antigens are more effective than monomeric antigens in inducing rearrangement of MHC class II compartments and antigen presentation by B cells [83]. Antigen valency and affinity also influence the differentiation of B cells into short-lived extrafollicular antibody-secreting plasma cells or to enter the germinal center. Decreasing antigen valency and affinity results in smaller numbers of antibody-secreting plasma cells, but does not affect the germinal center formation [84]. Thus, the property of antigens is an important factor that regulates B cell responses. However, little is known about how the BCR distinguishes and interprets differences in the structure and configuration of antigens.

The engagement of the BCR by antigens, particularly by multivalent antigens, has been shown to induce the reorganization of surface BCR. Early studies [249, 250] showed that

antigen cross-linking induced the redistribution of the surface BCRs to polarized caps. The formation of BCR caps required multivalent antigens [249, 251]. It has been shown that antigen cross-linking induced the association of the BCR with detergent-insoluble lipid rafts [252, 253]. Lipid rafts refer to membrane microdomains that are rich in cholesterol and glycosphingolipids [254, 255]. The BCR associated with lipid rafts is preferentially phosphorylated by Src-family kinases that are either constitutively associated or become associated with lipid rafts upon stimulation [19, 20, 22].

Downstream signaling molecules, like phospholipase  $C\gamma 2$ , are recruited to lipid rafts [256], but phosphatase CD45 is excluded [20]. These data support the model that the engagement of the BCR by antigens induces the formation of organized signaling microdomains on the B cell surface, and the lipid rafts provide platforms for BCR signaling microdomains. Batista et al. [51] provide strong evidence for such a model. In their report, it was demonstrated that BCR engagement by antigens tethered on the surface of target cells induced the redistribution of BCR, ganglioside  $G_{M1}$ , and tyrosine-phosphorylated proteins to the contact region between B cells and target cells. Adhesion molecules of the antigen tethered cell, like ICAM-1 and VCAM-1 and LFA-1 and VLA-4 of the B cell are recruited to the microdomains surrounding the BCR [52, 53]. CD45, CD22 and SHP-1 were excluded from this region of contact [257]. Such surface microdomains are similar to the immunological synapses formed between T cells and antigen presenting cells where MHC-restricted interaction is established and regulated [57]. However, the mechanisms for the formation of BCR signaling microdomains remain to be elucidated, and the factors that regulate BCR signaling microdomains remain to be identified.

The aim of this study was to investigate the role of antigen valency in regulating the cellular functions of BCR. Three different systems of model antigens (Fig. 3-1) and B cell models were used. Anti-Ig antibody based antigens were used with F(ab')<sub>2</sub> of anti-mouse IgM+IgG as a paucivalent antigen and biotinylated F(ab')<sub>2</sub> plus avidin as a polyvalent antigen (Fig. 3-1A). While anti-Ig antibodies have been widely used to experimentally induce B cell stimulation, they are not ideal antigens as they presumably bind to multiple epitopes on the BCR in contrast to 'real' antigen that binds only to the amino-terminal antigen-binding site. Therefore, antigens of different valences were also generated by conjugating phosphorylcholine (PC) to a carrier protein and hen egg lysozyme (HEL) to Ficoll (Fig. 3-1B); and used to study the effects of antigen valency on primary splenic B cells of mice expressing Ig transgene that is specific for PC or HEL. This work compares, for the first time the effect of these 'real' antigens with different valences on the formation of BCR surface signaling microdomains, recruitment of signaling molecules to these microdomains and intracellular trafficking of the BCR.

Increasing antigen valency is implicated in higher immunogenicity and is deemed a good strategy for developing better vaccines [258]. Vaccines that utilize multiple epitopes of antigens like viral antigens are more immunogenic requiring fewer booster doses and adjuvants. Smallpox, rubella and polio vaccines are examples of these. Hepatitis and rabies vaccines that contain monomeric subunits are less efficient in inducing B cell responses and require more booster doses [259]. Recent studies have shown that multiple repeating epitopes of single protein molecule can increase immune responses. Higher valency antigens induce a higher level of IgG antibodies than low valences of the same

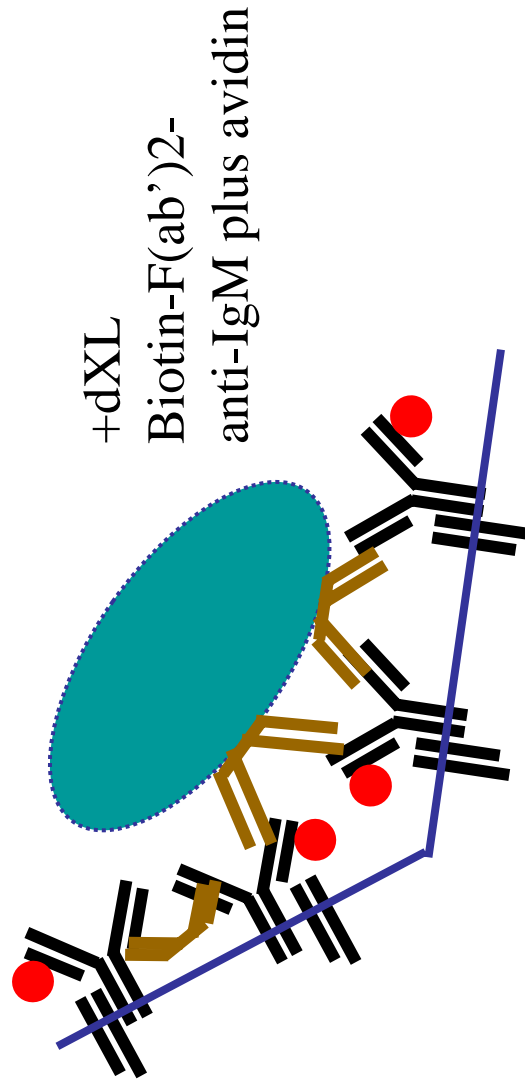
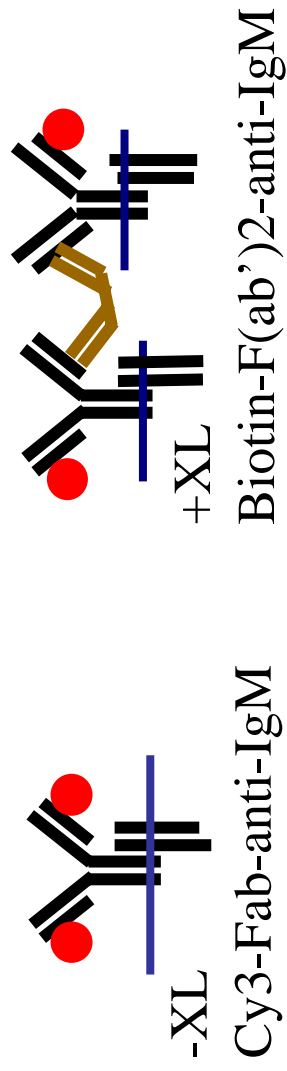
**Figure 3-1. Schematic representation of model antigens used.**

Shown is a representation of (A) BCR-specific antibody based antigens and (B) hen egg lysozyme (HEL) based antigens. (A) –XL indicates that surface BCR is labeled with fluorochrome tagged Fab fragments of anti-BCR antibody which label the surface BCR, but do not cross-link it. +XL and +dXL indicate that the surface BCR is cross-linked by paucivalent and polyvalent antigens, respectively. Biotinylated F(ab)'2 fragments of anti-BCR antibody and Biotinylated F(ab)'2 fragments of anti-BCR antibody followed by avidin are termed paucivalent and polyvalent antigens, respectively. (B) Hen egg lysozyme (HEL) forms dimers and trimers in solution and is a paucivalent antigen, while HEL conjugated to a Ficoll backbone serves as a polyvalent antigen.



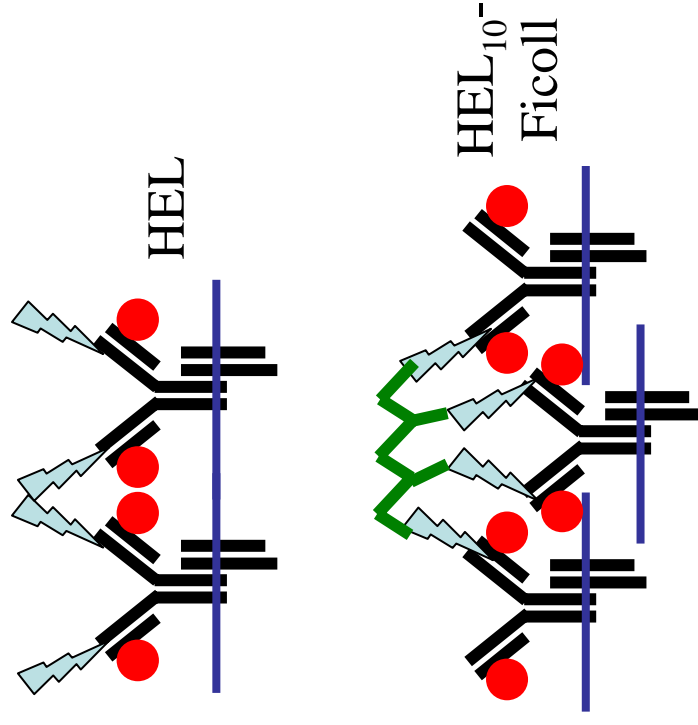
A

### BCR-specific antibody-based antigens



B

### Hen Egg Lysozyme-based antigens



antigens [260]. However the precise mechanism of the B cell response to varying antigen valences still remains unclear. Thus understanding the molecular and cellular responses of B cells stimulated with antigens of varying valences will lay the foundation for developing vaccines with better efficacy.

### 3.3 Materials and Methods

#### Cell lines and mice

The B cell lymphoma CH27 is an IgM<sup>+</sup>, H-2<sup>k</sup> and Fc $\gamma$ RIIB1<sup>-</sup> cell line. These cells express surface IgM that has been reported to bind phosphorylcholine [198]. CH27 cells were cultured at 37°C in DMEM supplemented as described previously [207] and containing 15% FBS.

Transgenic mouse strain C207-4, carrying the MOPC-167 (M167) myeloma-derived  $\mu$  plus  $\kappa$  transgene on a BALB/c background [261] was kindly provided by Dr. Michael Potter (NCI, NIH, Bethesda). This mouse expressed transgenic BCR specific for phosphorylcholine (PC). BALB/c mice were purchased from Charles River Laboratories (Frederick, MD). MD4 HEL transgenic mice on C57BL/6 background were provided by Dr. Susan Pierce (NIAID, NIH, Rockville). The MD4 mice carry the IghelMD4 transgene which recognizes hen egg lysozyme [262].

To obtain splenic B cells, single-cell suspensions of the splenocytes were prepared, and then subjected to Ficoll density gradient centrifugation (Sigma, St. Louis, MO) at 2300x g for 15 min. The mononuclear cell fraction was harvested and washed. T cells were depleted using anti-Thy 1.2 antibody and guinea pig complement. The B cells were washed and resuspended in medium. Monocytes and dendritic cells were removed by panning overnight at 37°C.

## **Antibodies and reagents**

Thy 1.2-specific monoclonal antibody (mAb) was obtained from BD Pharmingen (San Diego, CA). Guinea-pig complement and Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY). Hen egg lysozyme (HEL) and ovalbumin (OVA) were from Sigma Aldrich (St. Louis, MO). Amino-phenyl phosphoryl choline (APPC) and HEL<sub>10</sub>-Ficoll were purchased from Biosearch Biotechnologies (Novato, CA). Aldehyde activated dextran kit was obtained from Pierce Biotechnology (Rockford, IL). Cy3-conjugated Fab fragments of goat anti-mouse IgM, biotin-conjugated F(ab')<sub>2</sub> fragments of goat anti-mouse IgM+IgG and avidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). CD45-specific mAb and CD19-specific mAb were from BD Biotechnology, Inc. (San Diego, CA). 1D4B, a mAb specific for lysosomal associated membrane glycoprotein-1 (LAMP-1), anti-I-A<sup>b</sup> mouse mAb and TIB219, a mAb specific for mouse transferrin receptor (TfR) were obtained from hybridomas purchased from ATCC (Rockville, MD). TU-01, a mouse mAb specific for the alpha tubulin subunit of microtubules was purchased from Zymed Laboratories Inc. (San Francisco, CA). Anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). FITC-conjugated goat anti-mouse IgG<sub>2b</sub> was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). Alexa Fluor 488 conjugated- Cholera toxin B subunit was purchased from Molecular Probes (Portland, OR).

### **Serotyping M176 transgenic C207-4 litter mates**

To select for mice carrying transgenes of Ig specific for PC, the presence of anti-PC antibodies in the serum of M167 Tg mice was detected by ELISA as previously described [263]. Blood was collected by tail bleeds, and serum was titrated on PC-BSA coated plates. ELISA plates were coated with 100  $\mu$ l of PC-BSA (10  $\mu$ g/ml) in 50 mM carbonate/bicarbonate buffer was added to each microtiter well and left over night at 4°C. The plates were blocked with PBS containing 0.1% gelatin for 1 h at 37°C. Serum samples were diluted in the same buffer and incubated for 1 h at 37°C. Plates were washed three times with a buffer containing 0.01 M Tris, 0.14M NaCl, 0.05% Tween 20 buffer, pH 7.2. HRP-conjugated goat anti-mouse IgG antibody was added to the wells for 2 h at 37°C. The plates were washed and developed with 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in citrate buffer (pH 4.5) in the presence of 0.1% hydrogen peroxide. The plates were analyzed with a 96 well plate reader with OD at 405nm. The transgenic mice were defined as those animals whose sera had OD reading three times higher than wild type controls and litter mates.

### **Synthesis of PC –based antigens of varying valencies**

Phosphorylcholine (PC) was coupled to HEL and OVA as described by Shaw et al. [264]. Aminophenyl phosphorylcholine (APPC) (100 mg) was dissolved in 11 ml of 0.1 N HCl at 18 °C. NaNO<sub>2</sub> (27.7 mg) was added to the acid/APPC solution to activate APPC to form diazophenyl phosphorylcholine (DPPC). To conjugate with proteins like HEL and OVA, 100 mg HEL or OVA was dissolved in 50 ml borate-buffered saline pH 9.2 (10 mM borate, 0.15 M NaCl) and DPPC was added gradually and stirred overnight at pH 8.8.

The solution was dialyzed against phosphate-buffered saline (PBS) and sterile filtered. The protein concentration (HEL or OVA) in the solution was measured by the BCA Protein Quantification Kit (Pierce, IL). The number of moles of PC per mole of HEL or OVA was measured based on the excitation maximum and molar extinction coefficient of DPPC as previously described [265]. The molar extinction coefficient of DPPC at 475 nm is 12,600 in 0.1 N NaOH. By varying the ratio of DPPC to HEL or OVA in the above procedure, we were able to synthesize antigens of varying valencies. We synthesized HEL conjugated with 1, 2 or 4 moles of PC and OVA with 1, 3 or 5 moles of PC. PC<sub>5</sub>-OVA was also conjugated with dextran using an aldehyde activated dextran kit (Pierce Biotechnology, Rockford, IL) using the manufacturer recommended protocol. PC<sub>5</sub>-OVA-dextran helped to further increase antigen valency.

### **Antigen engagement of BCR**

Three different antigen systems were used in this study, including anti-mouse IgM antibody-based antigens (Fig.3-1A) , phosphorylcholine (PC)-based antigens and hen egg lysozyme (HEL)-based antigens (Fig. 3-1B). Splenic B cells from BALB/c or C57BL/6 mice or CH27 B cell lymphomas were incubated with 20 µg/ml of biotin-conjugated F(ab')<sub>2</sub> fragments of goat anti-mouse IgM+IgG (B-anti-Ig) as a paucivalent antigen (XL). For polyvalent antigen cross-linking (dXL), the BCR was incubated with 20 µg/ml of B-anti-Ig for 10 min followed by 20 µg/ml of avidin for an additional 20 min at 4°C before warming. Splenic B cells from M167 mice expressing BCR specific for phosphorylcholine, were incubated with 0.1 µg/ml of aminophenylphosphoryl choline (APPC) as a monovalent antigen. For polyvalent antigen cross-linking, the BCR was

cross-linked with equimolar concentrations of PC in the various conjugates. PC<sub>2</sub>-HEL (2.5 µg/ml) and 1.2 µg/ml of PC<sub>4</sub>-HEL were used to maintain the PC concentration at 0.3 nmoles. PC-OVA antigens (PC<sub>1</sub>, PC<sub>3</sub> and PC<sub>5</sub>) were used at 13.5 µg/ml, 4.5 µg/ml and 3 µg/ml respectively. Splenic B cells from the MD4 mice which express BCR specific for HEL were crosslinked with 6 µg/ml HEL as paucivalent or 22 µg/ml HEL<sub>10</sub>-Ficoll as polyvalent antigens. HEL has been shown to form dimers and trimers in solution [266]. All antigens were incubated with the cells for 30 min at 4°C to facilitate binding to the BCR, before warming to 37°C.

### **Immunofluorescence microscopy**

Cells ( $1 \times 10^6$ /ml) were incubated with 2.5 µg/ml of Cy3-conjugated Fab fragment of goat anti-mouse IgM (Cy3-Fab anti-IgM) to label the surface BCR. After 10 min, cells were incubated with model antigens for 30 min at 4°C. Cells were washed with DMEM containing 6 mg/ml of BSA (DMEM-BSA) to remove unbound antigen and incubated at 37°C for varying lengths of time at the end of the incubation. Cells were washed and adhered onto poly-L-lysine-coated slides (Sigma, St. Louis, MO) for 40 min at 4°C. Then, cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.05% saponin and incubated with antibody specific for LAMP-1 (1D4B) or alpha tubulin (TU-01) or phosphorylated ERK or phosphorylated JNK and FITC-conjugated secondary antibodies. For detection of phosphotyrosine-containing proteins, cells were incubated with anti-phosphotyrosine mAb (4G10) followed by FITC-conjugated anti-mouse-IgG<sub>2b</sub> secondary antibody. For labeling the surface ganglioside G<sub>M1</sub>, cells were incubated with AlexaFluor 488-conjugated cholera toxin B subunit (CTX-B) at 4°C before fixation. For labeling

surface molecules like, MHC class II, CD19, CD45 and transferrin receptor (TfR) cells were fixed and then incubated with antibodies against I-A<sup>b</sup>, CD19, CD45 and TfR respectively, followed by appropriate secondary antibodies. Cells were mounted with Gel/Mount (Biomedex, Foster City, CA). Slides were analyzed by laser-scanning confocal microscopy (LSM 510, Zeiss) using 100 x oil immersion objective. The same settings on the confocal microscope were used for all conditions of each set of experiments. Control experiments where the primary antibodies were either omitted or substituted by isotype control antibodies showed no significant staining signals.

For the quantitative analysis, one hundred cells in five images randomly taken from slides of each of three independent experiments were counted for each time point and condition. The number of cells containing polarized BCR caps was counted and expressed as a percentage of the total number of cells in the images.



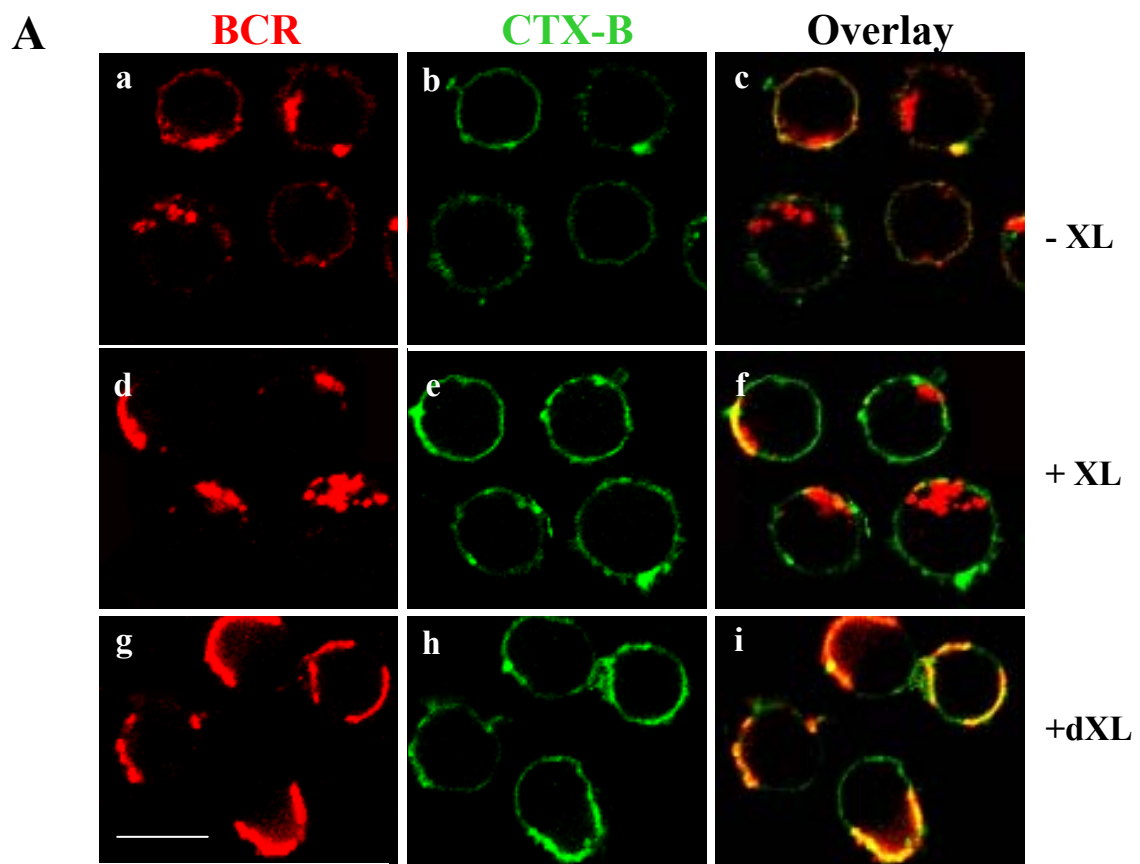
### 3.4 Results

#### **Antigen valency and the formation of BCR surface microdomains.**

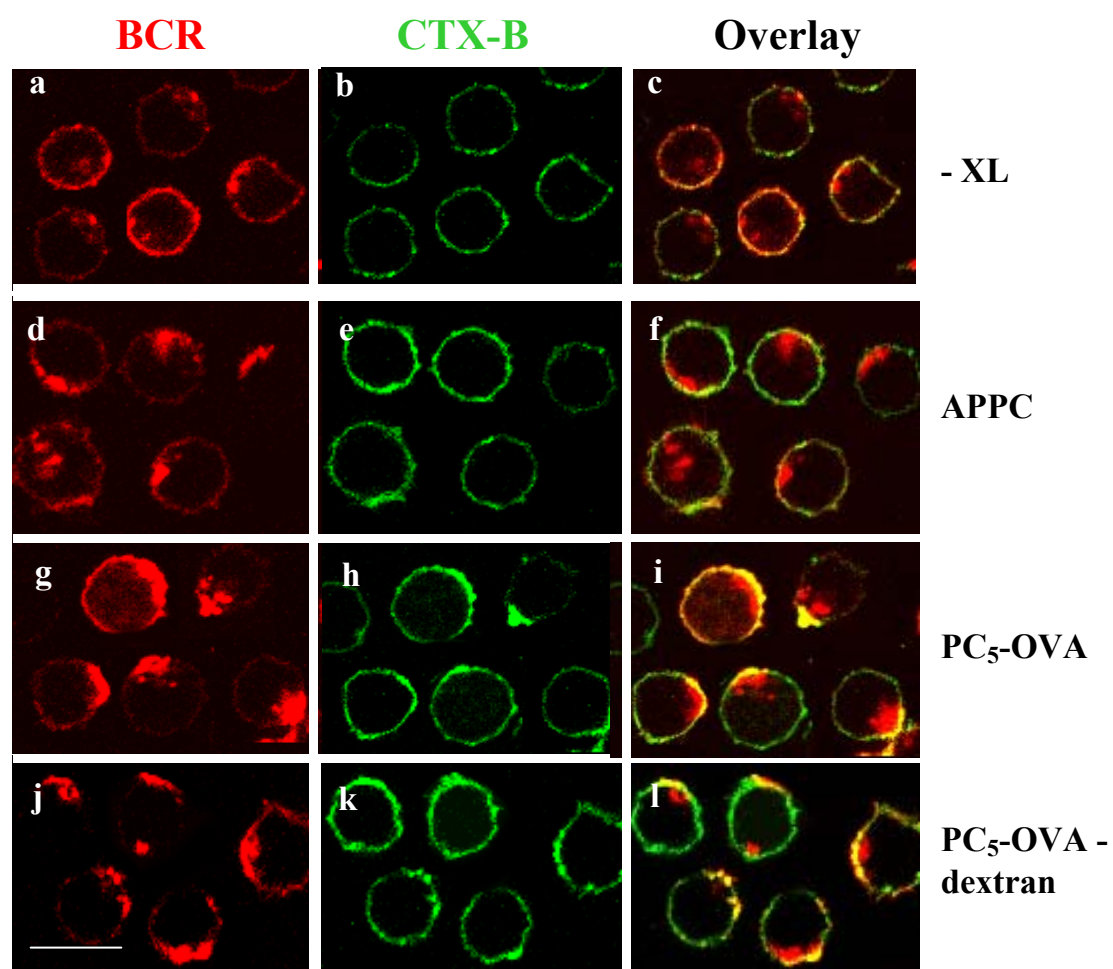
Upon cross-linking by antigens, the BCR translocates to lipid rafts where it organizes its signaling and endocytosis machinery [20, 253]. To determine the effect of antigen valency on the spatial relationship between the BCR and lipid rafts, the cellular distribution of the BCR and lipid rafts was analyzed using immunofluorescence microscopy. For these studies anti-BCR antibody-based antigens and phosphorylcholine (PC)-based antigens were used. To cross-link the surface IgM of splenic B cells from BALB/c or C57BL/6 mice using anti-BCR antibody-based antigens, biotin-conjugated F(ab')<sub>2</sub> fragments of goat anti-mouse IgM+IgG (B-anti-Ig) were used as a paucivalent antigen, and B-anti-Ig plus avidin was used to mimic a polyvalent antigen. To cross-link the BCR of splenic B cells from M167 transgenic mice, aminophenyl phosphorylcholine (APPC) was used as a monovalent antigen and phosphorylcholine conjugated to ovalbumin (PC-OVA) or hen egg lysozyme (PC-HEL) as polyvalent antigens. Cholera toxin B subunits (CTX) that bind to glycosphingolipid G<sub>M1</sub> with high affinity were used to label the lipid rafts. The surface BCR was labeled with Cy3-conjugated Fab fragment of goat anti-mouse IgM antibody (Cy3-Fab anti-IgM) at 4°C in the presence or absence of model antigens. As previously shown, B-anti-Ig does not compete with Fab-anti-IgM to bind to the BCR [219, 247]. After the chase at 37°C for 60 min, cells were cooled to 4°C and incubated with Alexa Fluor-488-CTX without permeabilization to label the surface G<sub>M1</sub>. In the absence of cross-linking, a portion of the surface labeled BCR moved into the cells while some remained on the cell surface, and CTX had a relatively homogenous

**Figure 3-2. Effect of antigen valency on the cellular distribution of BCR and ganglioside G<sub>M1</sub>**

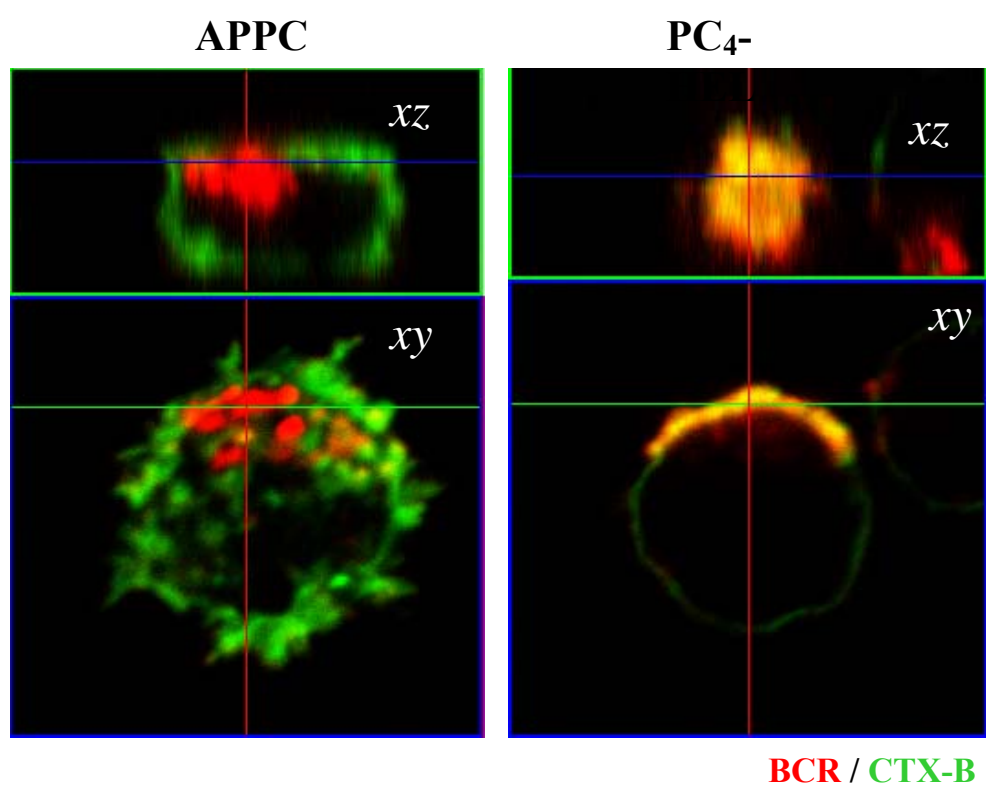
(A) Splenic B cells from BALB/c mice were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of 20 µg/ml B-anti-Ig (+XL) or 20 µg/ml B-anti-Ig plus 20 µg/ml avidin (+dXL) for 40 min at 4°C. (B and C) Splenic B cells from M167 transgenic mice were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of 0.1 µg/ml APPC or 3 µg/ml PC<sub>5</sub>-OVA or 2 µg/ml PC<sub>5</sub>-OVA conjugated to dextran (B) or 1.2 µg/ml of PC<sub>4</sub>-HEL (C) for 40 min at 4°C. After incubating at 37°C for 60 min, the cells were cooled for 10 min at 4°C, and stained with Alexa Fluor 488-CTX for 40 min at 4°C. Cells were then fixed and mounted. Images were acquired in the middle of the cells using a confocal fluorescence microscope. Bar, 5 µm (C) Shown is a representative image of the top view (xz) and side view (xy) of the BCR surface microdomain. On the left, is an image of a B cell from M167 transgenic mouse treated with APPC and on the right is a B cell treated with PC<sub>4</sub>-HEL.



**B**



C



surface staining pattern (Figure 3-2Aa-Ac and 3-2Ba-Bc). Cross-linking the BCR with B-anti-Ig alone increased the amount of BCR staining in the perinuclear area, but had no significant effect on the CTX staining pattern (Figure 3-2Ad-Af and 3-2Bd-Bf). Treating cells with B-anti-Ig and avidin or PC<sub>5</sub>-OVA or PC<sub>5</sub>-OVA conjugated to dextran resulted in a redistribution of the BCR, forming surface microdomains, while a small portion of the BCR was detected inside the cells. Significantly, in these cells, G<sub>M1</sub> staining was preferentially co-localized with the BCR (Figure 3-2Ah, 3-2Bh and 3-2Bk).

The surface BCR redistributed to a polarized, crescent appearance (Figure 3-2Ag) in response to BCR cross-linking. Such a polarized distribution of the surface BCR has been described as BCR caps [85, 249, 250]. Figure 3-2C shows a top view (xz) and side view (xy) of the BCR surface microdomain formed in cells treated with PC<sub>4</sub>-HEL (Fig. 3-2C, right panel) in comparison with a similar view of a cell treated with APPC (Fig. 3-2C, left panel). This showed that in B cells treated with PC<sub>4</sub>-HEL, the surface BCR moved together with the G<sub>M1</sub> to form a microdomain covering nearly one-third of the cell surface. Compared to anti-BCR antibody-based antigens, PC-based antigens induced the formation of BCR caps to a lesser extent (Fig. 3-2Bg). The colocalization of CTX as lipid raft marker and BCR indicates that both anti-BCR antibody-based and PC-based antigens induced the formation of BCR surface microdomains.

### **Effect of antigen concentration on formation of BCR surface microdomains**

To test the effect of antigen concentration on the formation of BCR surface microdomain, splenic B cells from M167 transgenic mice were treated with varying concentrations of PC<sub>2</sub>-HEL. The cells were incubated with Cy3-Fab anti-IgM at 4°C in the presence of

varying concentrations of PC<sub>2</sub>-HEL and chased at 37°C for varying lengths of time. After the chase, the cells were labeled for surface G<sub>M1</sub> at 4°C. Using immunofluorescence images, the percentage of cells containing polarized BCR surface microdomains was quantified. While there was a very slight decrease, there was no significant difference in the percentages of cells forming the polarized BCR microdomains with increasing antigen concentrations (Figure 3-3). This indicates that optimal concentration of PC<sub>2</sub>-HEL antigen lies in the range of 2 – 5 µg/ml of PC<sub>2</sub>-HEL, which corresponds to about 0.3 nmoles of PC. PC-based antigens of varying valences were titrated to maintain this molar concentration of phosphorylcholine in the following experiments.

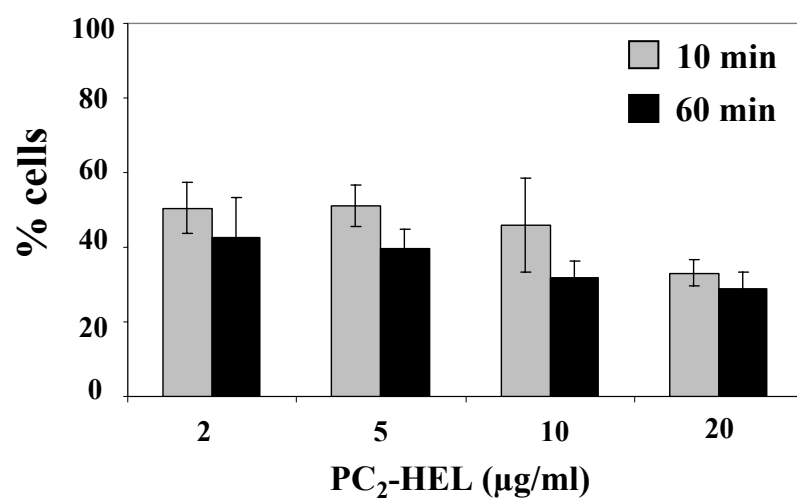
#### **Antigen Valency and the Time Course of BCR Surface Microdomain Formation.**

To follow the time course of the surface BCR redistribution, splenic B cells from M167 and MD4 transgenic mice were incubated with Cy3-Fab anti-IgM at 4°C in the presence of corresponding antigens and chased at 37°C for varying lengths of time. After the chase, the cells were labeled for surface G<sub>M1</sub> (Figure 3-4A and 3-4D). Before the incubation at 37°C, the BCR and CTX had a relatively homogenous surface-staining pattern in B cells treated either with the paucivalent or polyvalent antigen (Figure 3-4Aa, 3-4Ab, 3-4Da and 3-4Db). Upon warming to 37°C, distinct BCR microdomains were detected in few cells treated with APPC or HEL after 10 and 30 min incubation at 37°C and a majority of the G<sub>M1</sub> (Figure 3-4Ac, 3-4Ae, 3-4Dc and 3-4De) staining was co-localized with the BCR. By 30 and 60 min the BCR moved from the cell surface to the perinuclear location in

**Figure 3-3. Formation of surface signaling microdomains in response to varying concentrations of antigens.**

Splenic B cells from M167 transgenic mice were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of varying concentrations of PC<sub>2</sub>-HEL antigen for 30 min at 4°C, warmed up to 37°C for varying lengths of time and cooled for 10 min at 4°C. Cells were stained with Alexa Fluor 488 -CTX for 40 min at 4°C. Images were acquired in the middle of the cells using a confocal fluorescence microscopy. Bar, 10 µm. Shown is a quantitation of immunofluorescence images generated above. Five images containing 100 cells were randomly taken from slides of three independent experiments. The number of cells with surface signaling microdomains were counted for each time point and condition, and expressed as a percentage of the total number of cells in the images. Shown are the averages ( $\pm$  S.D.) of three independent experiments.

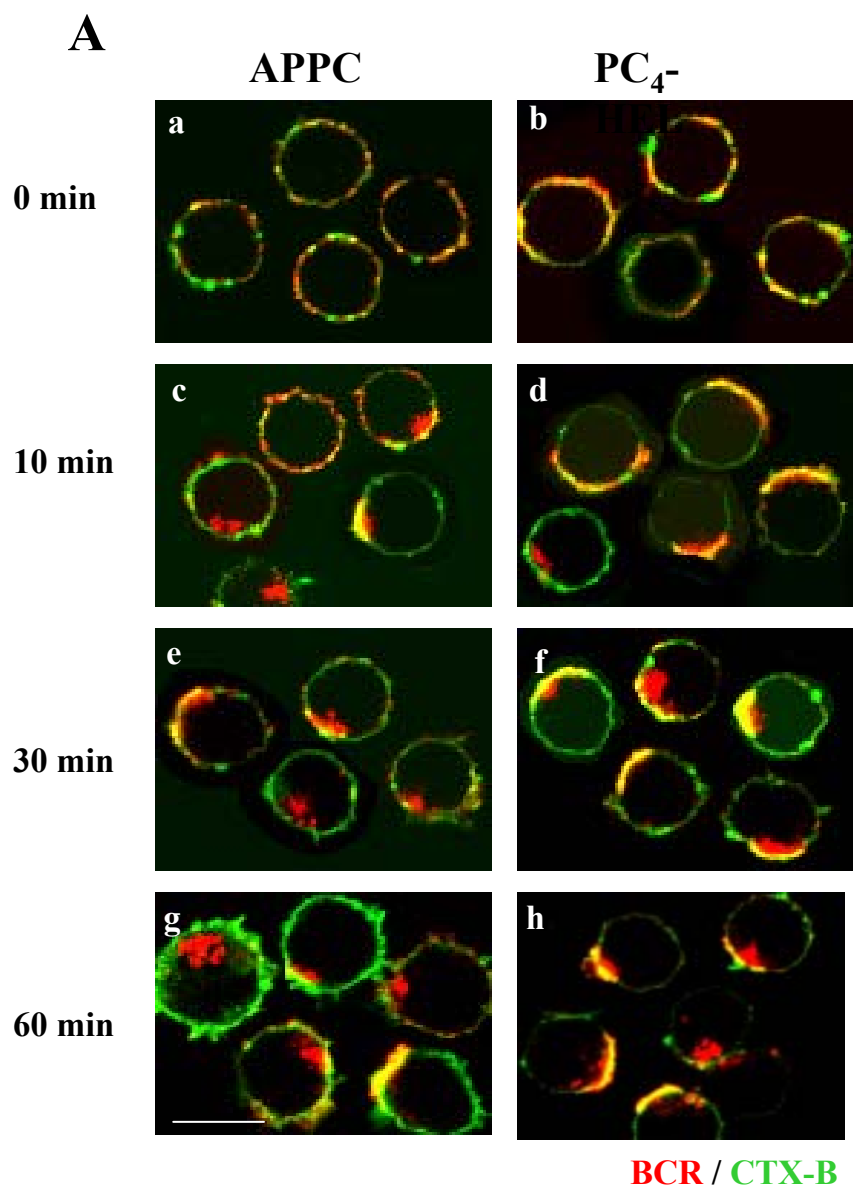


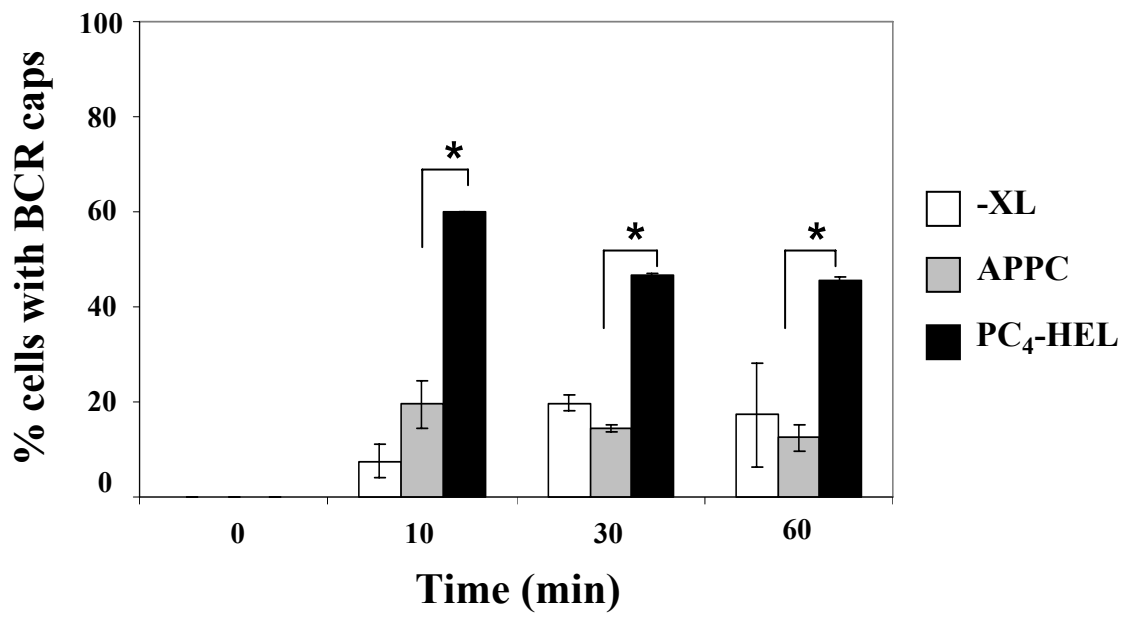
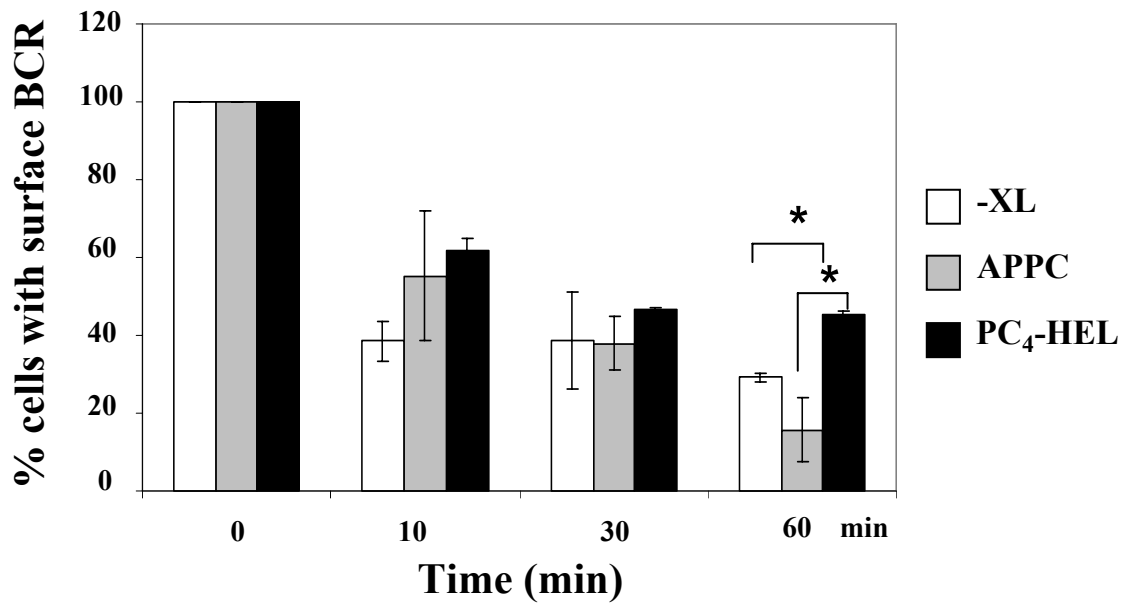


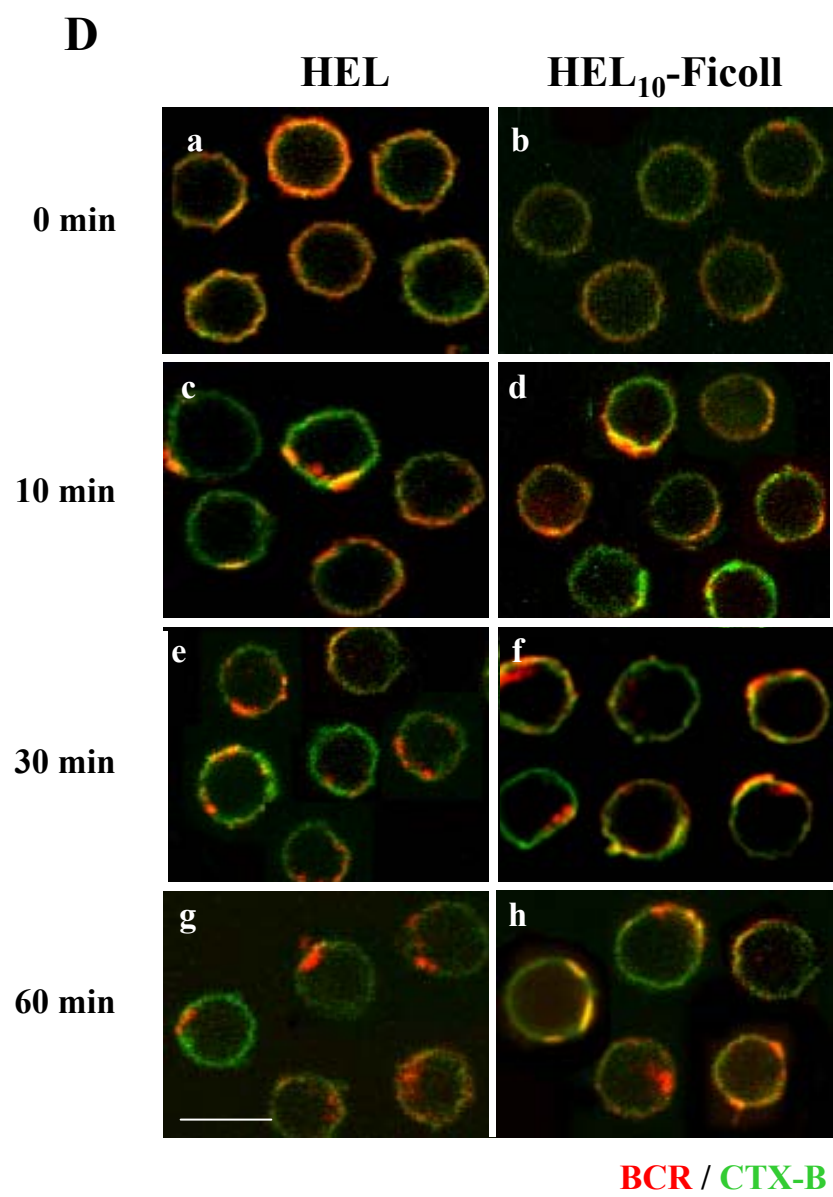
**Figure 3-4. Time course of formation of BCR surface signaling microdomains.**

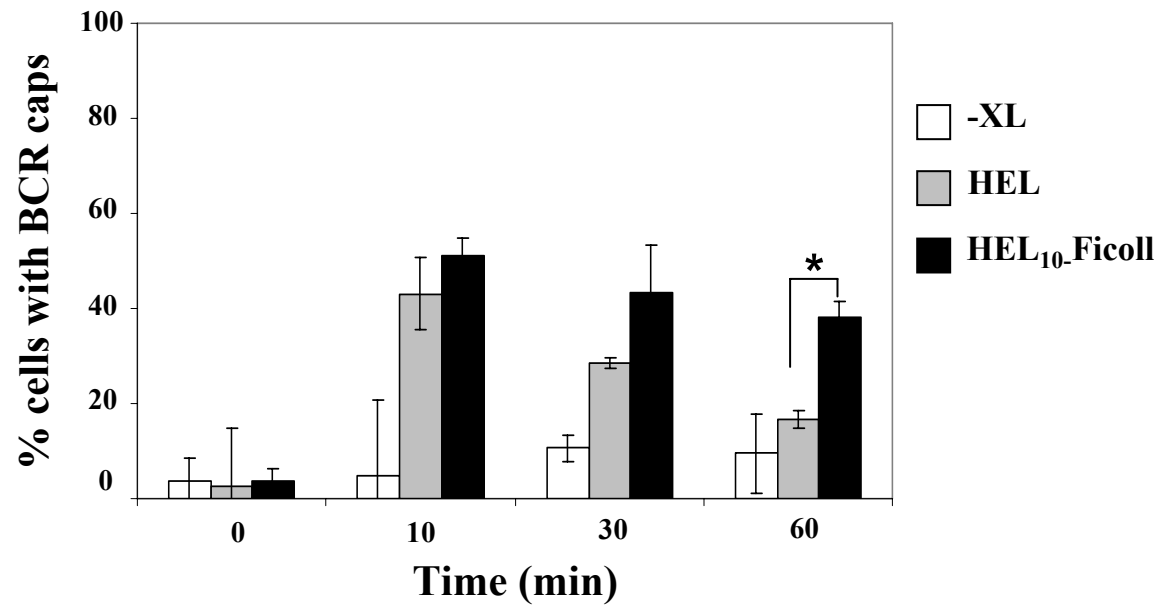
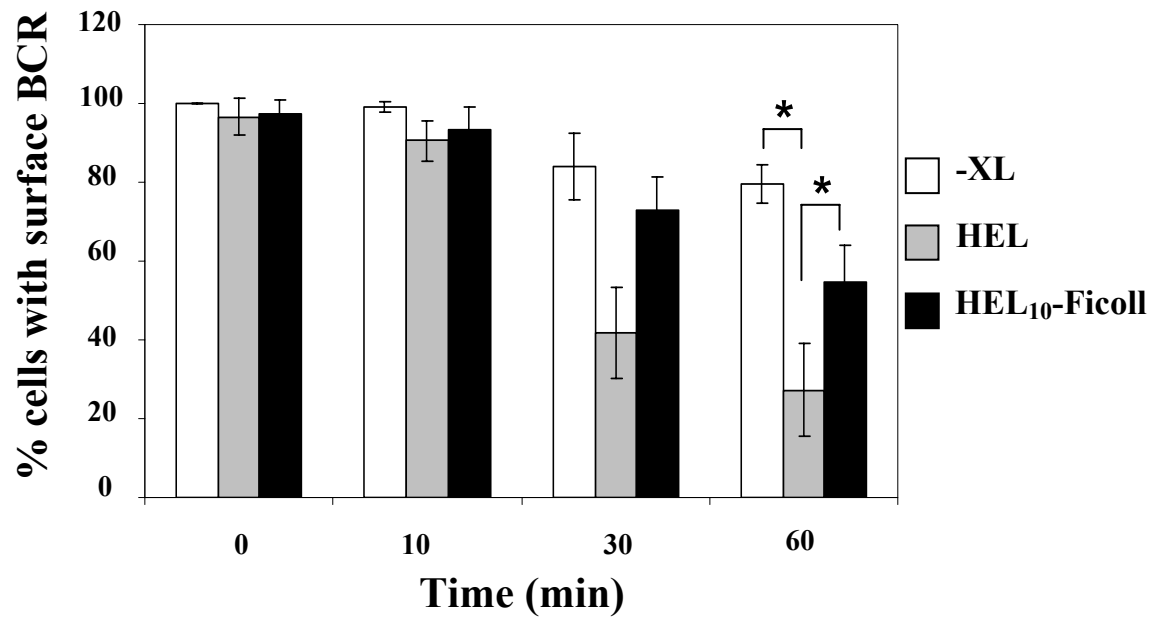
Mouse splenic B cells from M167 transgenic mice (A) and MD4 transgenic mice (D) were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of antigens. Cells were incubated with (A) 0.1  $\mu\text{g/ml}$  APPC or 1.2  $\mu\text{g/ml}$  of PC<sub>4</sub>-HEL or (D) 6  $\mu\text{g/ml}$  of HEL or 22  $\mu\text{g/ml}$  of HEL<sub>10</sub>-Ficoll for 30 min at 4°C. After incubating at 37°C for varying lengths of time, the cells were cooled for 10 min at 4°C, and stained with Alexa Fluor 488 -CTX for 40 min at 4°C. Cells were then fixed and mounted. Images were acquired in the middle of the cells using a confocal fluorescence microscopy. Bar, 5  $\mu\text{m}$ . (B, C, E and F). Shown are quantitative analyses of immunofluorescence images of percentages of B cells from M167 (B and C) or MD4 transgenic mice (E and F) with polarized BCR signaling microdomains (B and E) or retaining BCR on the cell surface (C and F). More than five images were randomly taken from slides of three independent experiments. The number of cells with surface signaling microdomains or surface BCR were counted for each time point and condition, and expressed as a percentage of the total number of cells in the images. Shown are the averages ( $\pm$  S.D.) of three independent experiments.

\* indicates a statistically significant difference ( $P < 0.05$ ) in percentages of cells with (B and E) polarized BCR caps or (C and F) total surface BCR.



**B****C**



**E****F**

cells treated with APPC or HEL. In cells treated with PC<sub>4</sub>-HEL or HEL<sub>10</sub>-Ficoll, the surface labeled BCR and G<sub>M1</sub> (Figure 3-4Ad and 3Dd) co-clustered to form microdomains in the majority of cells as early as 10 min and remained at the surface as BCR caps for at least 60 min (Fig. 3-4B and 3-4E).

Using these immunofluorescence images, the percentage of cells containing BCR microdomains was quantified (Figure 3-4B and 3-4E). When treated with APPC only 20% of cells from M167 transgenic mice showed the formation of surface microdomains at 10 min, while 60% of cells treated with PC<sub>4</sub>-HEL formed the surface BCR microdomains. Over the time course tested, more cells treated with PC<sub>4</sub>-HEL than APPC retained these microdomains on the cell surface. While 50% of PC<sub>4</sub>-HEL-treated cells retained the surface microdomains, only 10% of APPC-treated cells retained them after 60 min of incubation (Figure 3-4B, 3-4C). Similarly, MD4 transgenic mice followed the same trend as the M167 transgenic mice. At 60 min of incubation, a higher percentage of MD4 Tg B cells treated with HEL<sub>10</sub>-Ficoll (40%) formed the surface BCR microdomains compared to 10% of cells treated with HEL (Figure 3-4E, 3-4F).

Thus, both paucivalent and polyvalent antigens induced the polarized distribution of surface BCR and formation of surface BCR cap which co-localized with G<sub>M1</sub>. However, the BCR microdomains induced by the polyvalent antigens persist on the cell surface much longer than those induced by the paucivalent antigen. It is significant that this trend is consistently observed in different strains of mice and different types of model antigens that were tested. The prolonged BCR surface microdomains were not only observed

when the anti-BCR antibody-based antigens, which do not necessarily bind the antigen-binding sites of the BCR, were used, but also with two BCR-specific model antigens that bind to the antigen binding site of the BCR.

### **Antigen Valency and the Recruitment of Signaling Molecules to BCR Surface**

#### **Microdomain.**

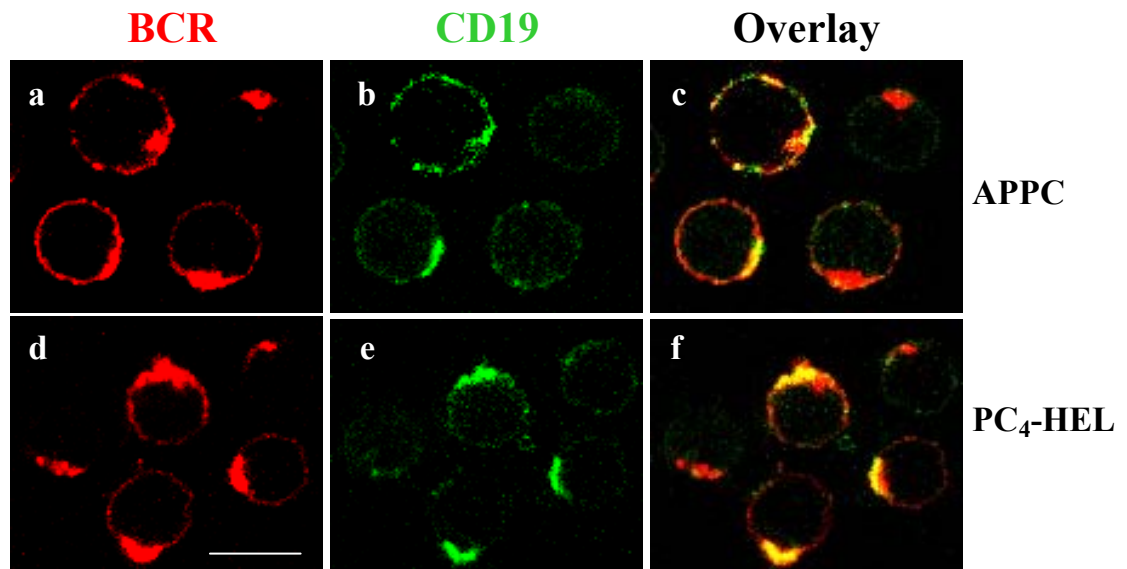
To determine whether antigen valency will affect the composition of the BCR surface signaling microdomains, we analyzed cellular distribution of signaling molecules in response to antigens with different valences. The cellular distribution of BCR co-receptor CD19, major histocompatibility complex (MHC) class II, phosphatase CD45, transferrin receptor (TfR), microtubules, tyrosine-phosphorylated proteins, phosphorylated extracellular signal regulated kinase (pERK), and phosphorylated c-Jun NH<sub>2</sub> terminal kinase (pJNK) in response to antigens of different valences were followed by immunofluorescence microscopy. Splenic B cells from M167 or MD4 transgenic mice or CH27 B cells were incubated with Cy3-Fab or FITC-Fab anti-IgM at 4°C to label the surface BCR, cross-linked with APPC, PC<sub>4</sub>-HEL, HEL, HEL<sub>10</sub>-Ficoll, B-anti-Ig or B-anti-Ig plus avidin, and chased at 37°C for 45 min. Tyrosine-phosphorylated proteins, phospho-ERK, phospho-JNK and microtubules were stained with respective monoclonal antibodies after fixation and permeabilization. CD19, MHC class II, CD45 and TfR – were labeled with specific antibodies 4°C without permeabilization to label surface proteins.



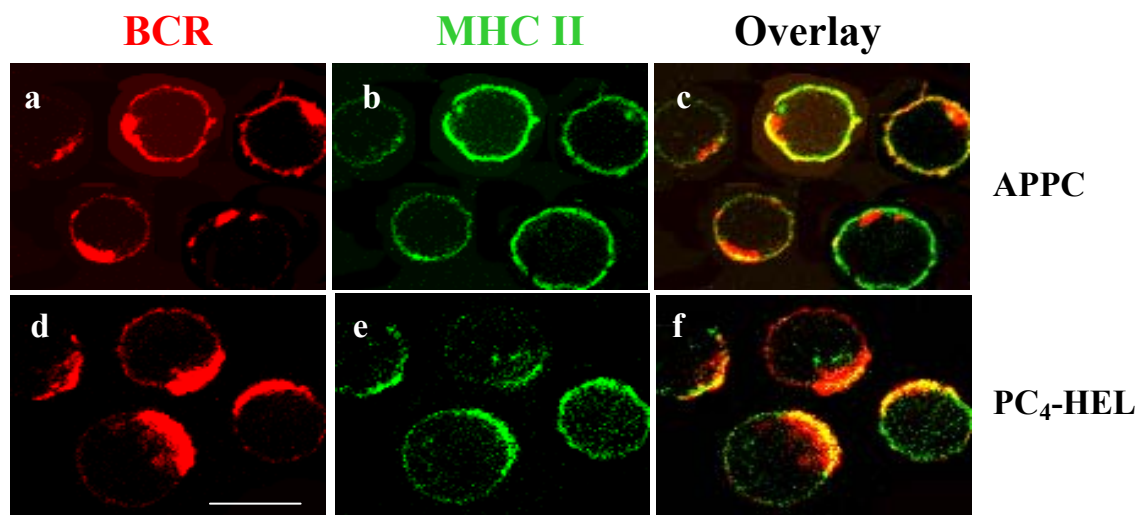
**Figure 3-5. Effect of antigen valency on recruitment of signaling molecules to BCR surface signaling microdomains.**

Splenic B cells from M167 transgenic mice (A-C and F), MD4 transgenic mice (G – J) and CH27 lymphomas (D, E and K) were incubated with Cy3-Fab or FITC-Fab anti-IgM in the presence of model antigens as described in *Materials and Methods*. After incubating at 37°C for 45 min, the cells were cooled for 10 min, and stained with mAbs specific for CD19 (A), I-A<sup>b</sup> (B), CD45 (C and D), TfR (E), tyrosine phosphorylated proteins (F), phospho-ERK (G and H) phosphor-JNK (I and J) or  $\alpha$ -tubulin (K) and secondary antibodies at 4°C. Cells were then fixed, mounted, and observed under a confocal fluorescence microscope. Bar, 5  $\mu$ m.

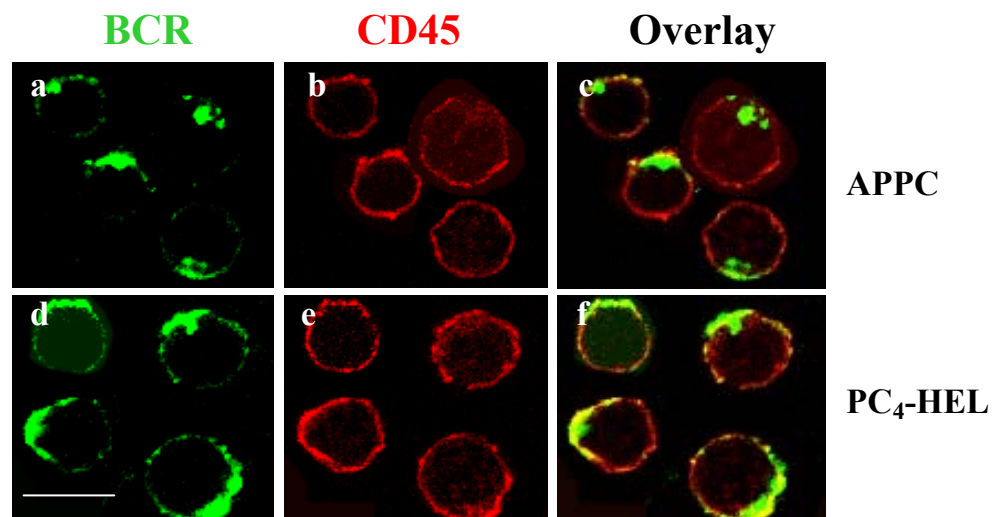
**A**



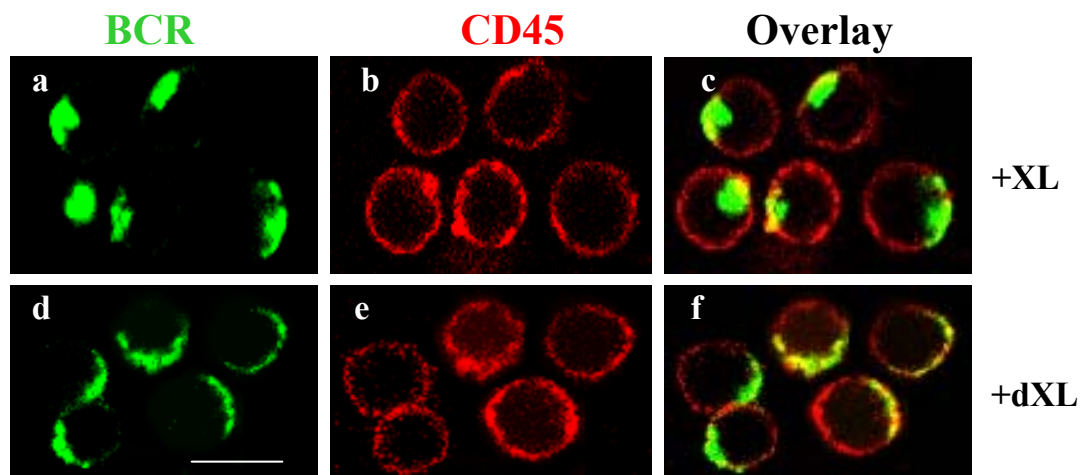
**B**



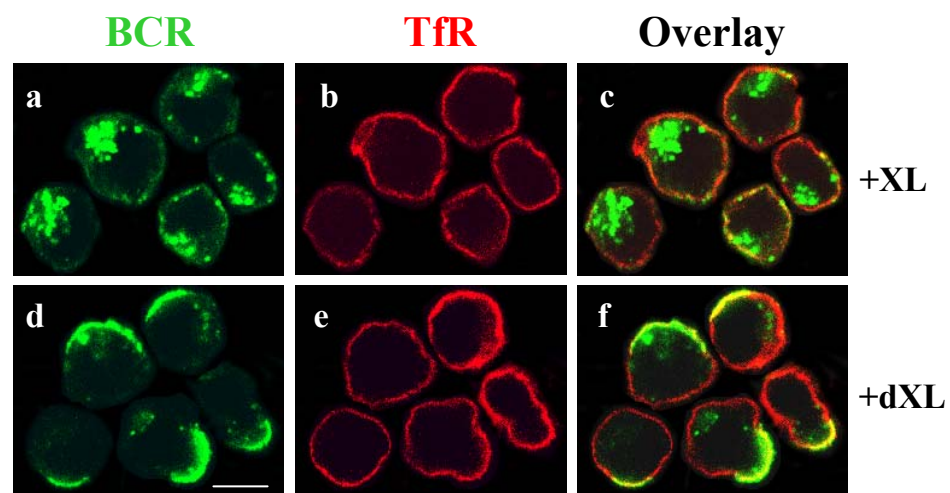
**C**



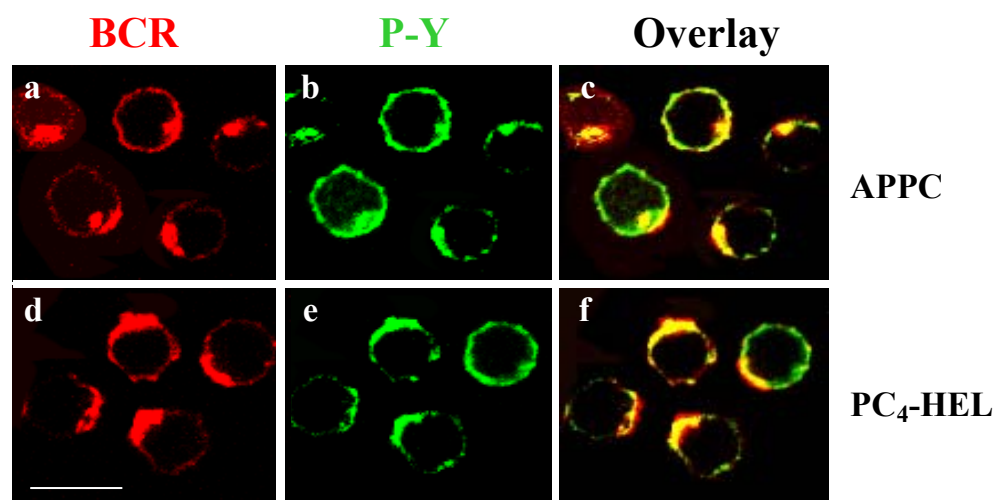
**D**

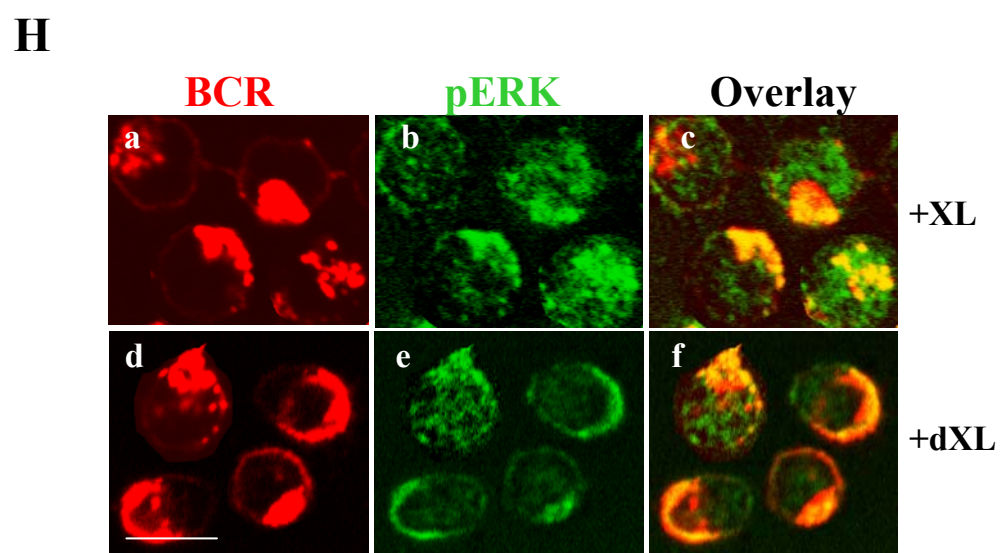
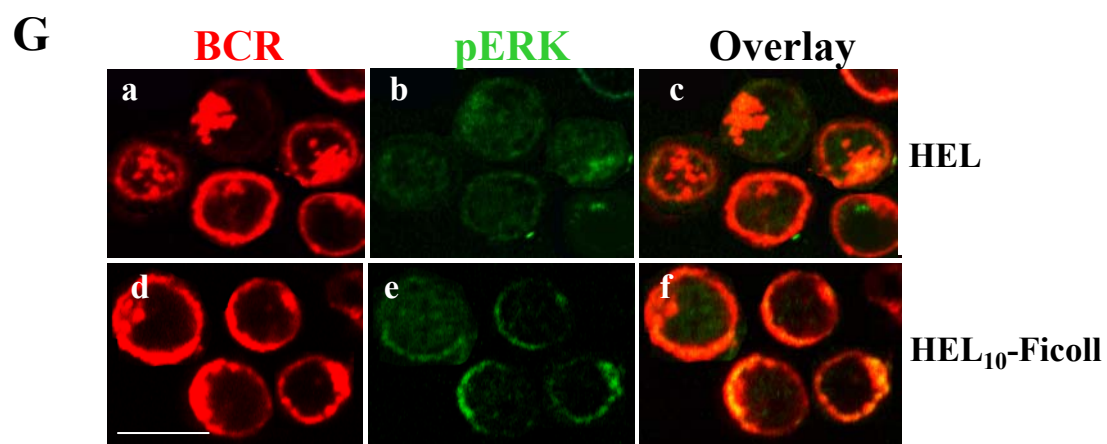


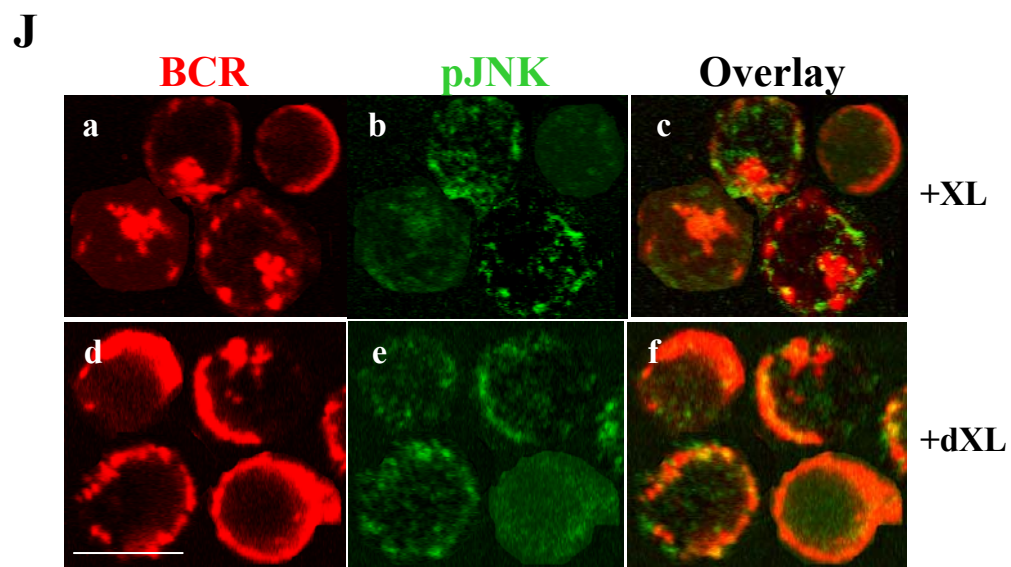
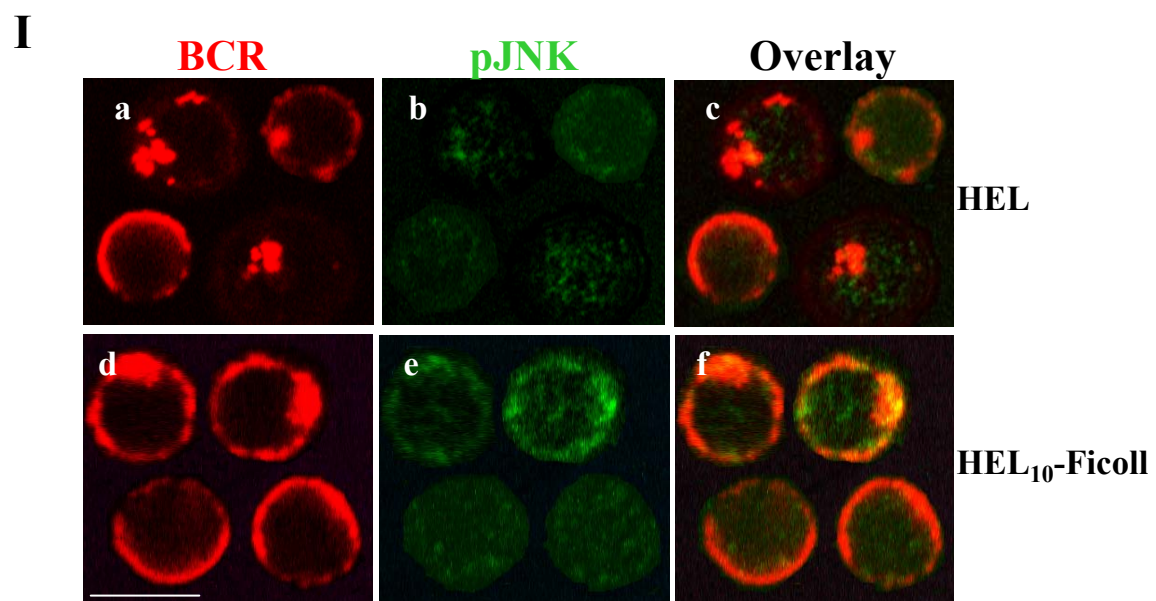
**E**



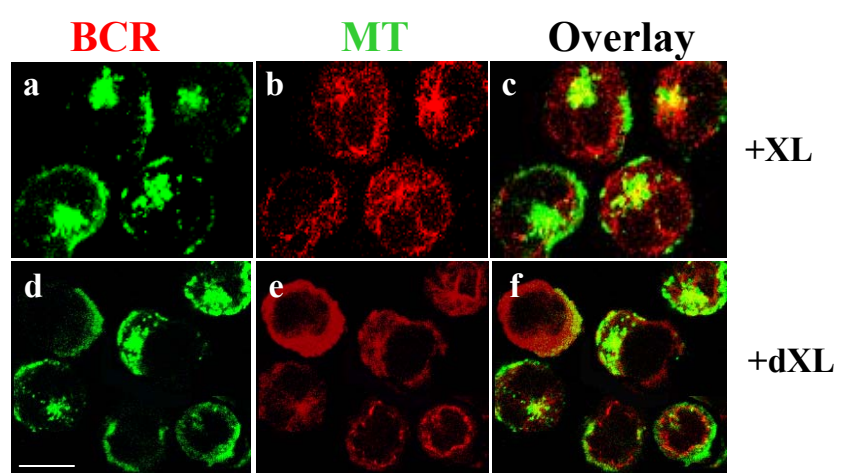
**F**







**K**



CD19 preferentially colocalized with the BCR surface microdomains in cells treated with APPC or PC<sub>4</sub>-HEL (Fig. 3-5A). This colocalization was more prominent in the polarized BCR surface microdomains formed in cells treated with PC<sub>4</sub>-HEL (Figure 3-5Ad-Af). MHC class II was evenly distributed on the surface of cells treated with APPC, but concentrated in the BCR surface microdomains when treated with PC<sub>4</sub>-HEL (Figure 3-5Ba-Bc). In contrast, CD45 (Figure 3-5C and 3-5D) and TfR (Figure 3-5E) staining appeared evenly distributed around the cell surface and neither the paucivalent nor polyvalent antigens had any significant effect on the surface distribution of CD45 and TfR. Significantly, CD45 and TfR did not concentrate in the polarized BCR caps. This indicates that the polyvalent antigen-induced BCR surface microdomains preferentially recruit CD19 and MHC class II but not CD45 and TfR.

Phosphotyrosine staining colocalized with the BCR in cells treated with APPC or PC<sub>4</sub>-HEL (Figure 3-5F). In cells treated with APPC, tyrosine phosphorylated proteins were colocalized with BCR on the surface and in the perinuclear location (Fig. 3-5Fa-Fc). PC<sub>4</sub>-HEL treated cells showed phosphotyrosine staining mainly on the surface and periphery of the cell, colocalizing with the polarized BCR surface microdomains (Figure 3-5Fd-Ff). In cells treated with HEL and B-anti Ig as paucivalent antigens, a majority of phosphorylated ERK and phosphorylated JNK is cytoplasmic and partially colocalized with the BCR in perinuclear location (Fig. 3-5Ga-Gc, 3-5Ha-Hc, 3-5Ia-Ic and 3-5Ja-Jc). Cells treated with HEL<sub>10</sub>-Ficoll and B-anti Ig followed by avidin, showed stronger phospho-ERK staining colocalized primarily with the BCR signaling microdomains (Fig. 3-5Hd-Hf and 3-5Hd-Hf). In contrast, phospho-JNK staining remains primarily



cytoplasmic with few cells showing colocalization of phospho-JNK staining with the BCR signaling microdomains (Fig. 3-5Id-If and 3-5Jd-Jf), in cells treated with polyvalent antigens. The recruitment of tyrosine phosphorylated proteins, active ERK and JNK suggests that the BCR surface microdomain formed by polyvalent antigens functions as BCR signaling microdomains.

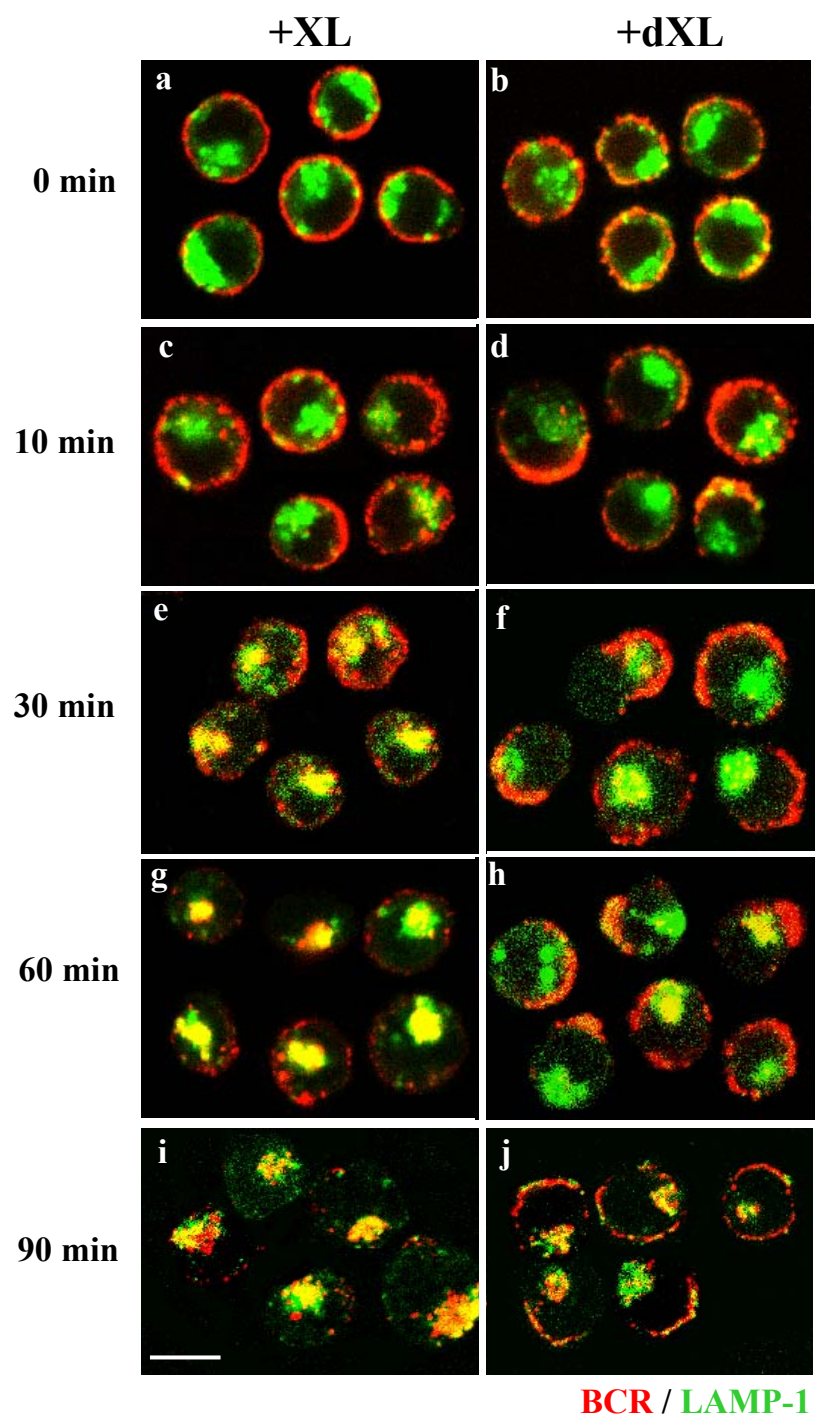
In cells treated with B-anti-Ig alone, the BCR co-localized with the microtubule organization center (MTOC) in the perinuclear region (Figure 3-5Ka -Kc). Upon cross-linking with B-anti-Ig and avidin, which induced the formation of BCR caps, the microtubules rearranged to the periphery of the cell and the MTOC was partially reoriented to the BCR caps (Figure. 3-5Kd-Kf). This suggests that polyvalent antigens altered the organization of microtubules in B cells.

### **Antigen Valency and the Intracellular Trafficking of the BCR**

To test whether antigen binding influences the kinetics of the movement of the BCR from the cell surface to late endosomes/lysosomes, the BCR on the surface of CH27 cells was labeled with Cy3-Fab anti-IgM in the presence of anti-BCR-based antigens of different valences and chased at 37°C for various times. The cells were labeled with anti-LAMP-1 antibody to mark the late endosomes/lysosomes. Similar to the results shown in Fig. 3-5A and 3-5D, before warming upto 37°C, a relatively homogenous surface-staining pattern of the BCR was observed (Fig. 3-6a and 3-6b) in cells either treated with B-anti-Ig alone or B-anti-Ig plus avidin. Upon warming to 37°C, the BCR in cells treated with B-anti-Ig appeared as punctate staining around the cell periphery (Fig. 3-6c), and then moved into

**Figure 3-6. Effect of antigen valency on movement of the BCR from cell surface to late endosomes/lysosomes**

CH27 cells were incubated with Cy3-Fab anti-IgM in the presence of 20 µg/ml B-anti-Ig (+XL) or B-anti-Ig plus avidin (+dXL) for 40 min at 4°C. Cells were washed and incubated at 37°C for varying lengths of time, and then fixed, permeabilized and incubated with mAb specific for LAMP-1 (1D4B) and FITC-conjugated goat anti-rat IgG secondary antibody. Images were acquired in the middle of the cells using a confocal fluorescence microscopy. Bar, 10 µm.



the cells (Fig. 3-6e). At 60 min, the majority of the BCR clustered in the perinuclear area and colocalized with LAMP-1 (Fig. 3-6g). Cross-linking of the BCR with B-anti-Ig and avidin resulted in the redistribution of the BCR to polarized caps and significantly reduced the colocalization of the BCR with LAMP-1 (Fig. 3-6f, h, j). While BCR caps persisted, the colocalization of the BCR with LAMP-1 in these cells increased over time (Fig. 3-6 f, h, j).

These data along with Figures 3-5C and 3-5F indicate that cross-linking the BCR with paucivalent antigens increases the movement of BCR to late endosomes/lysosomes. In contrast, cross-linking of the BCR with polyvalent antigens reduces the movement of the BCR from the cell surface to late endosomes/lysosomes.

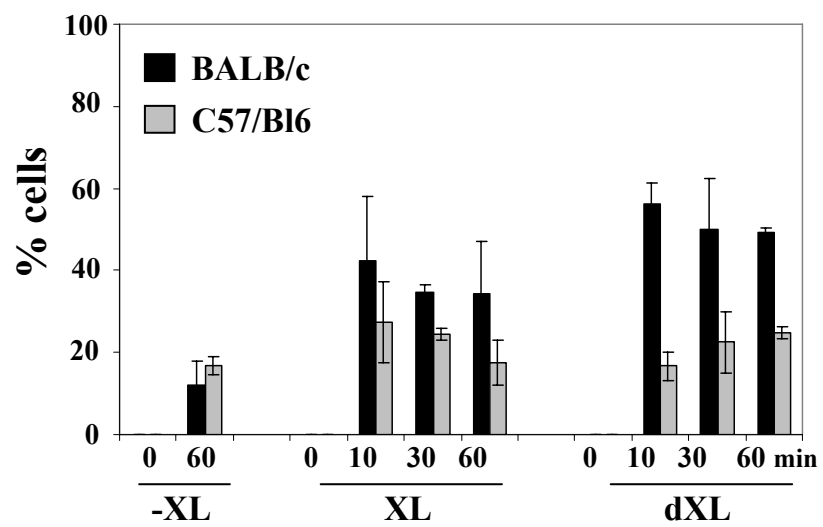
#### **Effect of different strains of mice and environment on the formation of BCR surface microdomains.**

We have used transgenic mice on BALB/c and C57BL/6 backgrounds in our study. To test whether intrinsic differences between the mice affect the ability of the B cells to form BCR surface microdomains, we compared the formation of BCR surface microdomains between BALB/c and C57BL/6 mice using immunofluorescence microscopy. Cells were incubated with Cy3-Fab anti-IgM alone (-XL) to label surface BCR or with B-anti-Ig (+XL) or B-anti-Ig followed by avidin (dXL) at 4°C, warmed up to 37°C for varying lengths of time and labeled for surface G<sub>M1</sub>. Using immunofluorescence images the percentage of cells containing polarized BCR surface microdomains was quantified. When treated with B-anti-Ig, there was no significant difference between the percentages

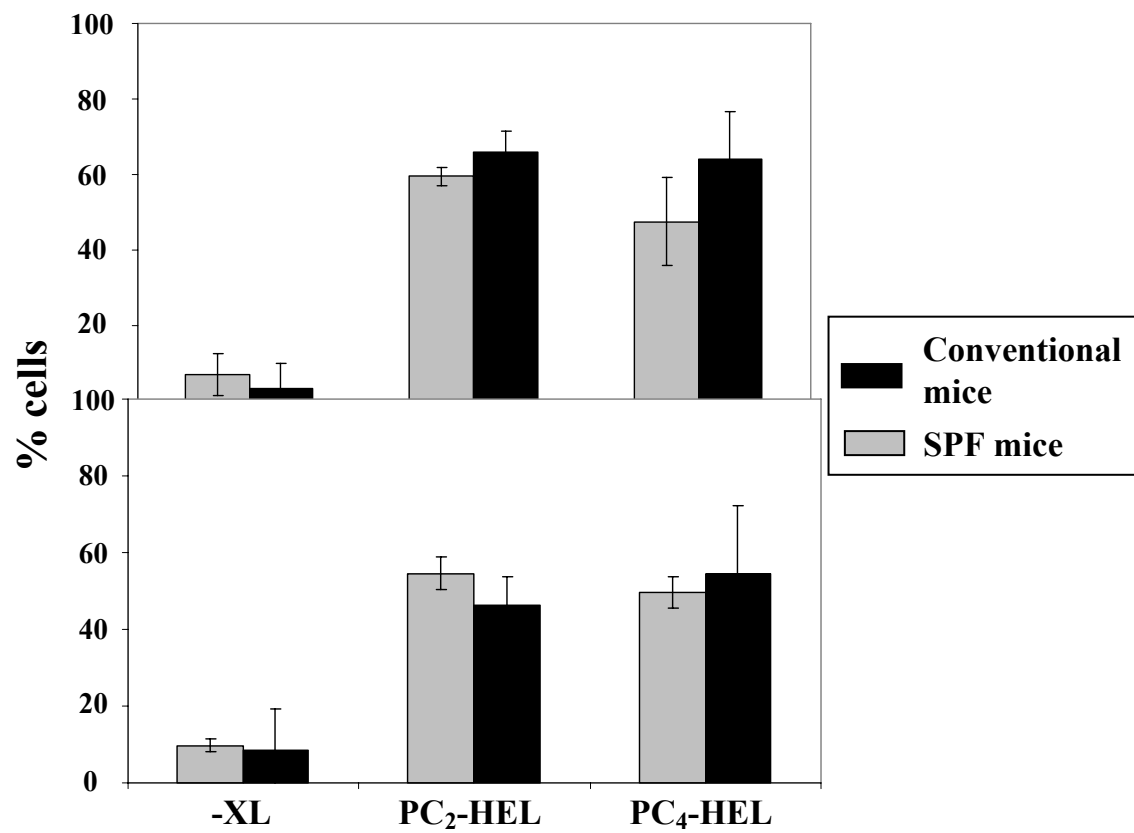
**Figure 3-7. Formation of BCR surface signaling microdomains as a function of mouse strain and environment.**

(A) Splenic B cells from BALB/c (black bars) and C57BL/6 (grey bars) mice were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of 20  $\mu\text{g/ml}$  B-anti-Ig (+XL) or 20  $\mu\text{g/ml}$  B-anti-Ig plus 20  $\mu\text{g/ml}$  avidin (+dXL) for 40 min at 4°C and then warmed up to 37°C for varying lengths of time. (B) Splenic B cells from SPF or conventionally housed C207-4 Tg mice were incubated with Cy3-Fab anti-IgM alone (-XL) or in the presence of 4.5  $\mu\text{g/ml}$  of PC<sub>1</sub>-HEL, 2.5  $\mu\text{g/ml}$  of PC<sub>2</sub>-HEL or 1.2  $\mu\text{g/ml}$  of PC<sub>4</sub>-HEL for 30 min at 4°C and then warmed up to 37°C for varying lengths of time. In both (A) and (B) the cells were cooled for 10 min at 4°C, and stained with Alexa Fluor 488 -CTX for 40 min at 4°C. Images were acquired in the middle of the cells using confocal fluorescence microscopy. Bar, 10  $\mu\text{m}$ . Shown is a quantitative analysis of cells containing surface BCR surface microdomains. Five images were randomly taken from slides of three independent experiments. The number of cells with surface signaling microdomains were counted for each time point and condition, and expressed as a percentage of the total number of cells in the images. Shown are the averages ( $\pm$  S.D.) of three independent experiments.

**A**



**B**



of splenic B cells from BALB/c and C57BL/6 mice showing the formation of BCR caps (Fig. 3-7A). However, when cells were treated with B-anti-Ig plus avidin, the percentages of BALB/c splenic B cells with BCR caps was significantly higher than that of C57BL/6 splenic B cells at all time points (Fig. 3-7A). This suggests that differences between the strains of mice affect the ability of B cells to form surface BCR microdomains.

Phosphorylcholine is a ubiquitous chemical present on the cell walls of many microorganisms that are found in the environment, like *Streptococcus pneumoniae* [48]. M167 transgenic mice spontaneously secrete PC-specific antibodies and the number of PC-positive B cells could be affected by the environment that the animals are housed in [267]. Hence, we compared the ability of splenic B cells from mice housed in specific pathogen free (SPF) and conventional environments to form BCR surface microdomains in response to antigens of different valences. Cells were incubated with Cy3-Fab anti-IgM alone at 4°C (-XL) or in the presence of equimolar concentrations of different valences of PC-HEL, warmed up to 37°C for varying lengths of time and labeled for surface G<sub>M1</sub> at 4°C. Using immunofluorescence images the percentage of cells containing polarized BCR surface microdomains was quantified. Similar to results shown in Figure 3-4B, cross-linking the BCR with PC antigens increased the percentages of cells that formed surface BCR microdomains after 10 and 60 min of incubation. However, there was no significant difference in the percentages of cells forming surface BCR microdomains in B cells from SPF or conventionally housed M167 transgenic mice (Figure 3-7B), indicating that B cells from SPF and conventionally housed M167

transgenic mice formed BCR microdomains with similar frequency when treated with PC-based antigens of different valences.



### 3. 5 Discussion

Considered together, the results presented here showed that both paucivalent and polyvalent antigens induce the formation of BCR surface signaling microdomains. Increases in antigen valency prolongs the lifetime of the surface signaling microdomains and recruits molecules involved in BCR signaling and antigen presentation to these microdomains. Significantly, different model antigen systems and different B cell model systems all showed similar results, strongly supporting our conclusion that antigen valency influences formation and stability of BCR surface signaling microdomains.

It has long been observed that the binding of polyvalent antigens to the BCR induces the reorganization of the surface BCRs into segregated surface domains, called BCR caps [249, 250]. However, the significance of BCR caps in B cell activation is not well understood. Here we show that cross-linking the BCR with a polyvalent antigen not only induced the formation of polarized BCR caps, but also leads to the colocalization of the lipid raft marker, CTX, and cellular tyrosine-phosphorylated proteins with the BCR, thereby suggesting that BCR caps are signaling microdomains on the B cell surface. Our time course study following the cellular distribution of surface labeled BCR showed that BCR caps were formed within 10 min in cells treated with both paucivalent and polyvalent antigens. When treated with paucivalent antigens, the number of cells containing BCR caps decreased as BCR internalization increased with time. The BCR moved into the cells more slowly and the BCR signaling microdomains persisted for a longer time in cells treated with polyvalent antigens. The surface signaling microdomains induced by antigens with relatively low valences were transient followed by BCR

internalization, while antigens with high valences stabilized the BCR signaling microdomains. The relationship between antigen valency and the lifetime of BCR signaling microdomains implies that modulating the stability of the BCR signaling microdomains is a mechanism by which antigens regulate BCR functions.

Antigen-induced BCR clusters that we report here appear to be similar to BCR synapses described by Batista et al. [51, 257]. Their study showed that B cell interaction with antigens attached to a target cell surface led to the concentration of the surface BCR in the contact region between the B cell and the target cell. The BCR along with CTX, tyrosine phosphorylated proteins, actin filaments and phospholipase C $\gamma$ 2 were found to accumulate in the region of the synapse, while the phosphatases SHP1 and CD45 were specifically excluded. Similarly, our data show that polyvalent antigens induced co-clustering of the BCR, CTX and tyrosine-phosphorylated proteins but not CD45. We also observed co-localization of CD19, MHC class II and phosphorylated ERK in the surface microdomains, indicating that these surface microdomains likely function as BCR signaling microdomains.

Different from the immunological synapses reported previously, the surface BCR clusters reported here are induced by soluble polyvalent antigens, but not antigens tethered on the cell surface. This extends the previous studies by demonstrating that soluble polyvalent antigens can induce the formation of stable surface signaling microdomains similar to the immunological synapses. However, the surface signaling microdomains induced by soluble antigens may be different from those formed in the contact region between two

cells, considering the ability of the BCR to distinguish antigens of different forms.

Indeed, we found that unlike the synapses formed in the cell-cell contact regions where CD45 is excluded, CD45 was neither preferentially concentrated in nor excluded from the polarized BCR caps induced by soluble antigens. Further studies are required to characterize BCR signaling microdomains induced by soluble antigens and compare them with those induced by antigens tethered on the cell surface.

Three different model antigen systems, including anti-BCR antibody-, phosphorylcholine- and hen egg lysozyme-based antigens, were used in this study. The antigens were designed and synthesized to change the number of BCR binding epitopes without altering the affinity and concentration. While anti-BCR antibodies have been widely used as surrogate antigens to induce B cell stimulation, they are not ideal model antigens as they presumably bind to multiple epitopes on the BCR in contrast to 'real' antigen that binds only to the amino-terminal antigen-binding site. Because they bind to different regions, it is possible that conformational changes that accompany antigen binding to the BCR are not replicated by cross-linking the BCR with anti-BCR antibodies. In addition to anti-BCR antibody-based antigens, in this study we have used PC- and HEL-based antigens to activate transgenic B cells that express BCR specific for PC and HEL. Phosphorylcholine is a ubiquitous chemical group found on the surface of Gram positive and lipopolysaccharide (LPS) of some Gram negative bacteria. For example, PC is expressed on the cell wall of *Streptococcus pneumoniae* [268]. The M167 transgenic strain of mice carries VH1/V $\kappa$ 24 gene segments of the myeloma protein MOPC-167 [261] and express BCR specific for phosphorylcholine [263]. The murine B cell

lymphoma CH27 has also been shown to bind synthetic phosphatidylcholine-containing lipid vesicles [198]. MD4 HEL transgenic mice express heavy and  $\kappa$  light chains specific for hen egg lysozyme [262]. Similar to anti-BCR-based antigens, both PC-based and HEL-based antigens induced the formation of stable BCR surface microdomains which colocalized with ganglioside  $G_{M1}$  and tyrosine phosphorylated proteins, and increases in valency of PC- and HEL-based antigens increased the stability of BCR surface microdomains, validating their use as model antigen systems.

It should be noted that the transgenic mice used are on two different genetic backgrounds and could influence the reproducibility of these data. We compared the levels of BCR surface signaling microdomains formed in B cells from BALB/c and C57BL/6 strains using anti-BCR antibody-based antigens. We found that there were higher percentages of B cells from BALB/c mice than C57BL/6 mice forming surface BCR caps in response to polyvalent antigens. Previous studies of capping response of B lymphocytes from different strains of mouse showed quantitative and kinetic differences in BCR cap formation [269] and the genetic differences between strains of mice have been speculated to give rise to such differences. Thus the differences between BALB/c and C57BL/6 mice should be considered when interpreting data from the different transgenic mice.

Our previous report showed that increases in antigen valency, which increased BCR residency in the surface signaling microdomains, enhanced the overall level and duration of protein tyrosine phosphorylation [85]. Here, we showed that CD19 co-localized with the BCR in signaling microdomains in cells treated with both paucivalent and polyvalent

antigens. However, this co-localization was more prominent in cells treated with polyvalent antigens. CD19/CD21 is a co-receptor complex that synergistically enhances BCR signaling by decreasing signaling threshold [270]. Recruitment of CD19 to the BCR signaling microdomains could be the underlying mechanism for enhanced signaling induced by polyvalent antigens. Furthermore, polyvalent antigens, but not paucivalent antigens induced the redistribution of surface MHC class II to co-localize with the BCR signaling microdomains. The biological significance of the recruitment of MHC class II to the BCR signaling microdomains remains to be elucidated. MHC class II has been shown to be preferentially located in the lipid rafts [271] and can transduce signals through Ig $\alpha$  upon engagement by TCR [272]. Recruitment of MHC class II to BCR signaling microdomains may provide it an opportunity to interact with Ig $\alpha$  and to contribute to BCR-mediated signaling. In addition, recruitment of MHC class II to BCR surface signaling microdomains could be a mechanism for the interaction of BCR signaling pathway with its antigen processing pathway. ERK and JNK are downstream MAP kinases of BCR signaling cascade. Syk and BLNK activate ERK through the Ras/Raf/MEK pathway [273], and JNK through Vav and Rac [274]. The MAP kinases phosphorylate and activate different sets of transcription factors including, Elk-1 and c-myc by ERK and c-Jun and ATF-2 by JNK. Phosphorylated, active form of ERK was also seen to co-cluster with the BCR in the surface signaling microdomain induced by polyvalent antigens, further enhancing signaling from the microdomains. However, there is partial co-localization of phosphorylated JNK with the BCR microdomains, indicating that ERK and JNK are differentially regulated in the B cells. This also reflects on the role of JNK is a stress-activated protein kinase, the activation of which has been implicated

during BCR-mediated apoptosis [275]. In contrast, CD45 was not concentrated in the BCR signaling microdomains induced by polyvalent antigens. CD45 is a receptor phosphatase that can dephosphorylate inhibitory tyrosine phosphorylation sites of Src-kinases in the early stage of BCR signaling [276] and dephosphorylate the active Src-kinases and dampen later stages of BCR signaling [20]. The lack of co-clustering of CD45 with the BCR signaling microdomains could delay the downregulation of signaling from the BCR surface microdomains. Thus, increases in antigen valency could enhance BCR signaling by increasing stability of BCR surface signaling microdomains and by recruiting BCR co-receptor CD19, and BCR signaling molecules, like phosphorylated ERK, but not signaling downregulators like CD45. This provides a novel explanation for why high valent antigens are generally more immunogenic.

How antigens regulate the stability of BCR signaling microdomains remains to be elucidated. We have previously reported that the increase in BCR residency in the surface signaling microdomains and BCR-triggered protein phosphorylation was associated with a decrease in BCR internalization [85]. This is consistent with our earlier observation that an inhibition of BCR internalization slowed down the attenuation of BCR-triggered protein tyrosine phosphorylation [219]. Here, we further showed that increases in antigen valency slowed down the movement of BCR from the cell surface to late endosome/lysosomes. These results suggest that inhibition of internalization is one of the mechanisms for stabilizing surface signaling microdomains. This reduced internalization is probably the result of large sizes of antigens, which become unmanageable for the endocytosis machinery. Additionally, the actin cytoskeleton that

has been shown to accumulate under BCR caps [277] could prevent the endocytosis machinery proteins from entering the signaling microdomains or inhibit membrane deformation required for endocytosis. Hence we observed that microtubules were partially reoriented to the BCR signaling microdomains or redistributed to the periphery of the cell in cells treated with polyvalent antigens. The accumulation of microtubules could also influence the internalization rate of the BCR and its movement from cell surface to late endosome/lysosomes.

In addition to internalization, the stability of BCR signaling microdomains is likely to be regulated by multiple interrelated factors. CD19 co-receptor was concentrated in the BCR signaling microdomains in cells treated with polyvalent antigens. Co-ligation of CD19 and BCR has been reported to prolong the residency of BCR in rafts [266], suggesting that interaction between BCR and CD19 in lipid rafts can contribute to the stability of the BCR surface microdomains. Syk has been shown to play a role in the formation of tightly capped BCR complexes on the cell surface [278], probably by regulating the assembly of actin filaments. Pure and Tardelli [78] reported that capping of the BCR in cells treated with the tyrosine kinase inhibitors genistein or tyrphostin was incomplete and retarded, which suggests a positive feedback from BCR-initiated signaling to BCR capping. Therefore, the recruitment of co-receptors and signaling component to the signaling microdomains and reorganization of the cytoskeleton could all influence the stability of BCR signaling microdomains.

The findings presented here may help to explain how the BCR distinguishes antigens with different properties, particularly structural differences. A major characteristic of T cell-independent type-2 (TI-2) antigens is their multiple, repeating antigenic determinants. TI-2 antigens include the outer membrane molecules displayed on the surface of microbial pathogens, such as antigens displayed on the surface of *Neisseria meningitis* and influenza that cause severe diseases [48]. B cell responses to TI-2 antigens are essential for immune protection against microbial pathogens. Self-antigens, like double stranded DNA, actin filaments, collagens and cell surface molecules, are often presented in polyvalent forms. Unresponsiveness of B cells to self-antigens is critical for immune tolerance. TI-2 antigens need to activate B cells in the absence of T cell help. Without signals provided by T cells, signaling initiated by the interaction of antigens and the BCR probably becomes the major driving force for B cell activation, and B cell responses to TI-2 antigens are likely to be sensitive to the magnitude and duration of the signaling events. The binding of polyvalent antigens stabilizes the surface signaling microdomains and recruits signaling molecules, like CD19 and ERK which provides a way to enhance BCR signaling and ensure B cell activation in the absence of T cell help. Besides BCR-initiated signaling and signals provided by T helper cells, additional signals provided by T cell-independent sources, such as Toll-like receptors and complement receptor CD21, are important for the outcome of antigen-BCR interaction [45, 48]. These additional signals can be triggered by surface molecules of microbial pathogens, DNA and complement factors associated with pathogens. In the absence of additional signals from either T cells or T cell-independent sources, extensive cross-linking of the BCR by polyvalent antigens has been shown to induce apoptosis [80, 81], which maintains the



self-tolerance of B cell responses. The relationship between the stability of B cell surface signaling microdomains and the fate of B cells remains to be examined. We can predict that both antigen structural properties and co-stimulatory signals regulate the stability and composition of BCR surface signaling microdomains, which influence the fate of B cells. Future studies should focus on detailing the differences in the composition of BCR surface signaling microdomains induced by antigens with different valences and cellular responses induced by these antigens.

## **Chapter 4: General Conclusions and Future Experiments**

### **4.1 Differential responses of primary B lymphocytes and B lymphoma cells to TLR9 ligand**

#### ***Conclusions***

The mammalian innate immune system recognizes conserved molecular patterns among microorganisms through the family of Toll-like Receptors (TLRs) [5]. Activation of the TLRs leads to induction of inflammatory responses and facilitates antigen-specific adaptive immunity [199]. TLR-9 recognizes DNA oligonucleotides (ODNs) containing unmethylated cytosyl guanosyl (CpG) sequences. The expression of TLR9 in humans is restricted to plasmacytoid dendritic cells and B cells [201]. TLR9 is differentially expressed and activated in subsets of B lymphocytes. Memroy B cells and transformed B cell lymphomas vary in their responses to CpG ODN compared to naïve splenic B cells. The work described in this dissertation characterized, for the first time, a potential mechanism for the differential responses of lymphoma B cells and primary splenic B cells to TLR9 ligand, CpG ODN.

In this dissertation, I have compared the differential effects of CpG ODN on mature splenic B cells and a B lymphoma cell line. Our studies show that CpG ODN induces proliferation of splenic B cells but leads to apoptosis of CH27 murine B lymphoma cell line. The apoptotic and proliferative effect of CpG ODN depends on the presence of the CpG motif. CpG ODN-induced apoptosis is mediated by TLR9 as a clonal variant of the B cell lymphoma line which does not express *tlr9* mRNA is unresponsive to CpG ODN.

This indicates that CpG ODN-induced apoptosis is a sequence-specific, TLR9 mediated event. Apoptosis in CH27 lymphomas was characterized by increased levels of cleaved caspase-3 and decrease of Bcl-xl, an anti-apoptotic protein.

TLR9 signaling leads to activation of NF- $\kappa$ B [146, 168]. CH27 B cell lymphoma line shows transient activation of NF- $\kappa$ B and degradation of I $\kappa$ B $\alpha$ , in response to treatment with CpG ODN. In sharp contrast, splenic B cells treated with CpG ODN exhibited sustained activation of NF- $\kappa$ B and degradation of I $\kappa$ B $\alpha$ . NF- $\kappa$ B inhibitor reduced the CpG-induced proliferation of splenic B cells and exacerbated the CpG-induced reduction in proliferation of CH27 lymphomas. These data indicate that CpG ODN-induced deregulation of NF- $\kappa$ B activation is a likely mechanism of apoptosis in the CH27 lymphoma cells. c-myc is constitutively expressed at high levels in CH27 B cell lymphomas. Treatment with CpG ODN leads to a further increase in c-myc protein levels followed by decrease to below constitutive levels in the B lymphoma cells while CpG ODN induced sustained increase in protein levels of c-myc in splenic B cells. Inhibitors specific for the MAP kinases, JNK, ERK and p38 did not affect the inhibitory effect of CpG ODN on CH27 cell proliferation, suggesting that MAP-kinases may not be involved in CpG ODN-induced apoptosis of CH27 lymphoma B cells. Taken together, these data indicate imbalanced activation of NF- $\kappa$ B and c-myc in the lymphoma B cells is a potential mechanism that leads to CpG-induced apoptosis.

The data presented here showed that CpG-induced apoptosis of CH27 B lymphoma cells occurs by deregulated NF- $\kappa$ B activation and c-myc and Bcl-xl expression. CpG ODN

induces proliferation of primary B cells in culture by sustained activation of NF- $\kappa$ B and by upregulating expression levels of c-myc and Bcl-xl. In contrast, CpG ODN induced apoptosis in lymphoma B cells due to an abortive activation of NF- $\kappa$ B, which could lead to the down-regulation of pro-proliferation genes like c-myc and anti-apoptotic genes like Bcl-xl. It was found that decrease in NF- $\kappa$ B activity was concurrent with an over-elevated c-myc level in CpG ODN-treated CH27 lymphoma B cells. Imbalance between the decreasing activity of NF- $\kappa$ B and high levels of c-myc could generate a situation, where cell proliferation induced by c-myc in the absence of sufficient anti-apoptotic signals from NF- $\kappa$ B, culminates in apoptosis. Therefore, CpG ODN-induced imbalance between c-myc and NF- $\kappa$ B is a likely underlying mechanism for CpG ODN-induced apoptosis.

Our data and previous studies suggest a potential therapeutic value for CpG DNA in cancer treatment. Cancers are treated based on the type, location and progression of the tumor. Traditional cancer therapies including radiation therapy and chemotherapy kill, shrink or block proliferation of tumor cells and have deleterious side effects on normal bystander cells. Further these treatments do not eliminate tumors completely resulting disease recurrence. Activating the host immune system can result in targeted deletion of tumor cells and holds great promise for cancer therapy. TLR9 agonist, CpG ODN has demonstrated potent antitumor activity in mouse models and human clinical phase trials.

Tumors can be treated with CpG ODN as monotherapeutic, as adjuvant in tumor vaccines or in combination with monoclonal antibodies or chemotherapy. Monotherapy of cervical

carcinoma and T lymphoma with CpG ODN show tumor regression in a T cell or NK cell-dependent manner [204]. TLR9 expressing cancer cells like malignant B cell chronic lymphocytic leukemias (B-CLLs) are sensitized to monoclonal antibody therapy without increasing toxicity to normal cells when treated with CpG ODN [279]. While there is a lot of enthusiasm for the use of vaccines against solid tumors it has translated into little success in clinical trials [280]. Induction of cytotoxic CD8<sup>+</sup> T lymphocytes (CTL) which can directly kill tumor cells is the goal of cancer vaccines. This depends on the activation of these cells through their interactions with CD4<sup>+</sup> T cells and antigen presenting cells like dendritic cells. Toll-like receptor agonists, TLR9 agonists in particular are promising candidates that can enhance the T cell response of cancer vaccines [281]. As adjuvant in cancer vaccines, CpG ODN can stimulate tumor-infiltrating plasmacytoid dendritic cells (pDCs) creating a Th-1 like cytokine and chemokine milieu. The activated pDCs up-regulate co-stimulatory molecules and present tumor antigens to CD4<sup>+</sup> T cells which can activate CTLs. CpG ODN in combination with tumor vaccines has been shown to be more effective in eradicating established tumors in mice [282]. Monoclonal antibody-based therapy is the fastest growing class of cancer therapy. Rituximab or Rituxan, an anti-CD20 monoclonal antibody has demonstrated efficacy in treating patients with various forms of lymphoid malignancies [283]. CpG ODN in combination with Rituximab monoclonal antibody enhances antibody mediated cytotoxicity of tumors [284] and with chemotherapy reduces suppressive effects of T regulatory cells, enhancing T cell response against tumors [285].

Our study further supports the use of CpG ODN in cancer treatment. The remarkable differential responses of primary B cells and B lymphoma cells to treatment with CpG ODN further indicates that CpG ODN can be used to eradicate TLR9 expressing cancer cells without toxicity to normal cells. CpG 7909 (also known as CpG 2006) has been administered to more than 400 patients in human clinical trials for lung cancer and melanoma [126]. As yet, there has been no report of organ cytotoxicity in these patients. Our data provides further evidence that targeted activation of TLR9 by CpG ODN in cancer cells that express TLR9 can tremendously enhance cancer treatment without harming normal cells.

Activation-induced cell death mediated by TLRs has been described a number of times and seems to be an evolutionarily conserved mechanism. Plant disease resistance genes, R genes, have LRRs and TIR domains and share homology with the TLR [286]. These genes initiate a hypersensitive response characterized by localized cell death, which is similar to apoptotic death, and helps limit the spread of microbes [287]. In mammalian cells, bacterial lipoproteins signaling through TLR2, LPS through TLR4, and dsRNA through TLR3 and CpG DNA through TLR9 have been shown to induce apoptosis especially in stimulated cells [185, 191, 196, 202]. While immunostimulatory functions of TLRs are well characterized, the mechanism and relevance of TLR-mediated apoptosis remains unclear. However, it can be speculated that apoptosis of activated cells serves to limit their lifespan and control the duration of the inflammatory response [186]. This is probably a crucial feedback mechanism, to prevent an overactive inflammatory response that could lead to autoimmune diseases and disease pathologies like sepsis and airway

inflammation [203]. Thus, the TLR9-mediated apoptotic pathway described in this dissertation can be speculated to be a physiologically relevant mechanism by which the mammalian host controls inflammation and induces an effective immune response.

### ***Future Experiments***

TLR9-induced cell death has been previously reported, however the underlying mechanism of this phenomenon has not been well characterized. We have described here a CpG-sequence specific, TLR9-mediated onset of apoptosis in a B lymphoma cell line. To further understand TLR9-induced apoptosis, as future work we will determine, which apoptotic pathway is activated in the cells activated by TLR9 ligand. Our data indicates that caspase-3 is involved in this response. Caspase-3 can be activated by caspase-8 or caspase-9. Caspase-8 mediates apoptosis signaling from the death receptor family (extrinsic apoptosis pathway) while caspase-9 is activated by release of cytochrome c from mitochondria (mitochondrial apoptosis pathway). Following the activation of these two caspases by Western blots and flow cytometry, will allow us to test whether the extrinsic or mitochondrial pathway is responsible for TLR9-induced apoptosis. If the extrinsic pathway is implicated in TLR9-induced apoptosis, we should next determine whether CpG ODN regulates the expression of death receptors and their ligands, like Fas, Fas ligand, TRAIL receptors, TRAIL, on the lymphoma B cells in comparison to splenic primary B cells. Further, we should test the functional involvement of FAS and TRAIL receptor using antibodies that are able to block ligand binding. In addition, we need to examine whether CpG ODN regulates the expression and activation of other Bcl-2 family members like Bok, Bad, Bim, Puma and Noxa, in addition to Bcl-2 and Bax.

MyD88, the primary adaptor molecule in TLR9 signaling pathway, contains a death domain (DD). In cells undergoing apoptosis through TLR2, MyD88 has been shown to bind Fas associated death domain protein (FADD) via DD-DD interactions [186], which activates caspase-8 leading to apoptosis. Recent studies have also implicated a role for FADD in TLR-induced proliferation of B cells. B cells from FADD-deficient mice do not undergo proliferation in response to stimulation with TLR3 and TLR4 ligands [288]. This suggests that FADD is involved in TLR signaling in B cells. The dual role of FADD in TLR-induced apoptosis and proliferation in B cells makes it an interesting candidate for further study. It will be interesting to test whether FADD is involved in CpG ODN-induced apoptosis in CH27 B lymphomas, using CH27 cells that are deficient in FADD. We can transfect dominant negative constructs of FADD into CH27 cells and test the response of these cells to CpG ODN. We can further compare the kinetics and levels of activation of FADD in the splenic B cells and CH27 B lymphoma cell. This will yield clues about possible differences in the TLR signaling pathway that leads to proliferation or apoptosis in the two types of B cells.

CpG ODN-induced apoptosis suggests a therapeutic effect of CpG ODN on cancers that express TLR9. To further test the therapeutic potential, we should test the effect of CpG ODN on human B cell lymphomas and patient biopsies of B cell lymphomas. From published studies, it is evident that TLR-induced apoptosis differs based on apoptotic machinery available and cytogenetic status of the cells [186, 195, 196]. B cell lymphomas are characterized into various types based on molecular alterations and only certain types



of B cell lymphomas are likely to respond to CpG ODN. Molecular profiling of patient biopsy samples of lymphomas that respond to CpG ODN will help to narrow the likely candidates that can be treated with CpG ODN.

## **4.2 Effect of antigen valency on BCR functions**

### ***Conclusions***

Humoral immune responses are initiated by antigen binding to the B cell antigen receptor (BCR). Binding of antigen to the BCR triggers a signaling cascade and initiates the internalization of the BCR-antigen complex. B cells are exposed to myriad antigens of varying affinity, size, valency, concentration, stereochemical structure and density of antigenic epitopes. The quality and quantity of immune responses mounted by B cells are modulated by these inherent properties of antigens. However, little is known about how the BCR distinguishes and interprets differences in the structure and configuration of antigens. The goal of this study was to investigate the role of antigen valency in regulating the function of BCR

In this dissertation, three different model systems of antigens and B cells were used to study the effects of antigen valency on BCR functions. Anti-BCR antibody based antigens, phosphorylcholine-based antigens and hen egg lysozyme-based antigens. Anti-BCR antibodies have been extensively used to induce B cell stimulation; however they are not ideal antigens as they presumably bind to multiple epitopes on the BCR in contrast to ‘real’ antigen that binds only to the amino-terminal antigen-binding site. Therefore, phosphorylcholine (PC)-based and hen egg lysozyme (HEL)-based antigens were used, which bind specifically to the antigen binding site of BCR of mice carrying Ig transgene specific for PC and HEL. Significantly, all three model systems of antigens and cells influenced BCR functions in a similar manner.

Antigen binding to the BCR induces a translocation of the BCR to lipid rafts where it initiates signaling cascades and organizes its endocytosis machinery that leads to the internalization of the BCR-antigen complex for antigen processing and presentation to T cells. My studies show that stimulating the BCR with both paucivalent and polyvalent antigens led to rearrangement of the BCR into polarized surface microdomains which co-localized with the lipid raft marker, ganglioside G<sub>M1</sub> and tyrosine-phosphorylated proteins, suggesting that these microdomains possibly function as signaling microdomains on the B cell surface. Surface signaling microdomains that are induced by polyvalent antigens persisted longer on the B cell surface than those induced by paucivalent antigens. This was also accompanied by a decrease in movement of the BCR to LAMP-1 positive late endosomal/lysosomal compartments. Thus antigen valency influences the formation and stability of the BCR surface signaling microdomains.

Our previous published study has shown polyvalent antigens enhance the level and duration of protein tyrosine phosphorylation [85]. In the present study, we observed that polyvalent antigens induced the concentration of signaling molecules like, CD19, MHC class II and phosphorylated form of MAP kinases, ERK and JNK in BCR signaling microdomains but not signaling down regulators like, CD45 or transferrin receptor. Thus, polyvalent antigen-induced formation of stable BCR surface signaling microdomains leads to recruitment of signaling molecules like, CD19 and phosphorylated ERK and JNK which enhance signaling from the BCR surface microdomains. We further propose that enhanced signaling from BCR microdomains could act as a positive feedback loop,

leading to the recruitment of proteins like the cytoskeletal proteins and CD19/CD21/CD81 complex, which further stabilize the microdomains.

Based on data presented here and previous studies [85], we propose that antigen valency influences BCR functions by regulating the formation and stability of BCR surface signaling microdomains and differentially recruiting signaling molecules to the microdomains. Paucivalent antigens cross-link the BCR and initiate signaling and internalization of the BCR. The BCR-antigen complex is internalized much more rapidly in cells treated with these antigens compared to cells treated with antigens that do not cross-link the BCR. The antigen-induced signaling from the BCR activates the transcription of genes that control B cell responses. Enhanced internalization of the BCR-antigen complexes leads to effective processing and presentation of antigens to T cells, which is essential for induction of T cell- dependent B cell responses. Polyvalent antigens form stable BCR signaling microdomains in lipid rafts and enhance signaling but lead to a reduction in internalization of the BCR-antigen complexes. The enhanced signaling from polyvalent antigen-induced BCR microdomains could elicit a B cell response, even in the absence of T cell help. Reduction in internalization and movement of the BCR-antigen complex limits the availability of T cell help. T-independent signals in the form of Toll-like receptors and complement receptors probably have a significant influence on the cellular responses of the cells exposed to polyvalent antigens. Thus we propose that antigen valency regulates the formation and stability of BCR surface signaling microdomains which influences cellular responses of the B cell.

Rational design of immunogens that are capable of eliciting protective immunity is a challenge in developing potent vaccines. Many variables of the antigen like valency and orientation affect the immunogenicity of vaccines. While there is evidence that increasing antigen density or valency results in enhanced humoral responses, few attempts have been made to compare how antigen specific B cells distinguish and respond to antigens of varying valences. Efficacy of conjugate vaccines like *Hemophilus influenza* B (Hib) vaccine which consist of purified capsular polysaccharide covalently linked to carrier protein are affected by length of polysaccharide chain and density of carbohydrate hapten [289]. Studies in monkeys and humans have shown that antibody titer increased with the number of repeating carbohydrate units [290, 291]. Zinkernagel and colleagues have shown the close correlation between epitope density and B cell responses in viral infections [292]. Recent studies have shown that multiple repeating epitopes of single protein molecule can increase immune responses [293]. Thus the work on the effect of antigen valency on BCR functions described in this dissertation seeks to define the effect of antigen valency on BCR functions. The data indicates that increasing antigen valency enhances and prolongs BCR signaling while reducing the internalization and trafficking of the BCR from cell surface, supporting the theory that increasing antigen valences can be an effective tool to develop vaccines with better efficacy.

### ***Future Experiments***

Upon antigen binding, the BCR translocates to lipid rafts, where it organizes signaling and endocytosis machinery and initiates signaling cascades and transport of antigen into the cell for processing and presentation. Our studies indicate that antigen properties

influence the formation and stability of BCR surface signaling microdomains. Future studies should focus on the underlying mechanism of the regulatory role of antigen valency on BCR functions and its relationship with antigen immunogenicity. To understand how antigen valency regulates BCR function, I would like to determine the effect of antigen valency on the composition of the BCR microdomains and the kinetics of recruitment of different molecules. Upon antigen binding, the BCR translocates to lipid rafts and signaling is initiated by Src family kinases like Lyn, Fyn, Blk. Signaling is further amplified by kinases, Syk and Btk [35]. Immunofluorescence microscopy can be used to study the effect of antigen valency on the recruitment of Src kinases, Syk and Btk to BCR signaling microdomains. Cellular responses of the B cells are influenced by the activation of MAP kinases and transcription factors. Hence it will be interesting to see the effect of antigen valency on total cellular phosphorylation levels, the phosphorylation of MAP kinases - JNK, p38 and ERK, and activation of transcription factors like NF- $\kappa$ B, AP-1, ATF, and c-myc. Antigen binding to the BCR also stimulates the internalization and trafficking of the BCR-antigen complexes for processing and presentation to T cell. Our data showed that polyvalent antigens cause a reduction in internalization of the BCR and movement to late endosomal/lysosomal compartments. This suggests that the endocytosis machinery proteins like clathrin, dynamin and AP-2 are regulated differentially when antigens of varying valences bind to the BCR. I would like to test the effect of antigen valency on the recruitment of clathrin, dynamin, AP-2 and the phosphorylation of clathrin. Further, it is speculated that the ITAMs of the Ig $\alpha$ /Ig $\beta$  heterodimers contain motifs that influence the internalization of the BCR [294]. Recent fluorescence resonance energy transfer (FRET) studies [19, 22] indicate that antigen

binding leads to conformational changes of the Ig $\alpha$ /Ig $\beta$  heterodimer. It can be hypothesized that antigens of different valences could induce different conformational changes in the Ig $\alpha$ /Ig $\beta$  heterodimer influencing the access of signaling molecules and endocytosis machinery proteins to the BCR. It will be interesting to test this with immunofluorescence microscopy and FRET studies. Upon antigen binding, the B cell mounts diverse cellular responses including, proliferation, cytokine secretion, differentiation to antibody secreting plasma cells, anergy or apoptosis. Studies have implicated that antigen valency influences the cellular responses [77, 80] of B cells. I would propose to test the effect of antigen valency on B cell responses *in vitro* and *in vivo*. *In vitro* studies will be carried out to examine the effect of antigen valency on proliferation of B cells, Ig and cytokine secretion and efficiency of antigen processing and presentation in B cells. *In vivo* experiments will monitor titers of antibody and generation of memory B cells in mice immunized with antigens of varying valences. It will be of significant interest to correlate the molecular signaling events to the cellular responses of proliferation, antigen processing and presentation, antibody secretion and memory B cell generation using a system of physiologically relevant antigens. Increasing antigen valency is implicated in higher immunogenicity and is deemed a good strategy for developing better vaccines [258]. Thus, understanding the cellular and molecular responses of B cells stimulated with antigens of varying valences lays the foundation for developing better vaccines.

## **Appendix A: Effect of CpG DNA on A20 and 38C13 B cell lymphomas**

This section describes the effect of CpG DNA on A20 and 38C13 mouse B cell lymphomas.

A20 is a mouse B cell lymphoma line expressing an IgG2a mouse BCR (H-2<sup>d</sup>, Fc $\gamma$ RIIB1<sup>+</sup>). A20 cells were cultured at 37°C in DMEM supplemented as described previously [207] and containing 15% FBS. The murine B cell lymphoma 38C13, which expresses surface IgM was grown as described by Woolridge et al. [295]. 38C13 has been extensively used to study monoclonal antibody therapy of tumors [295].

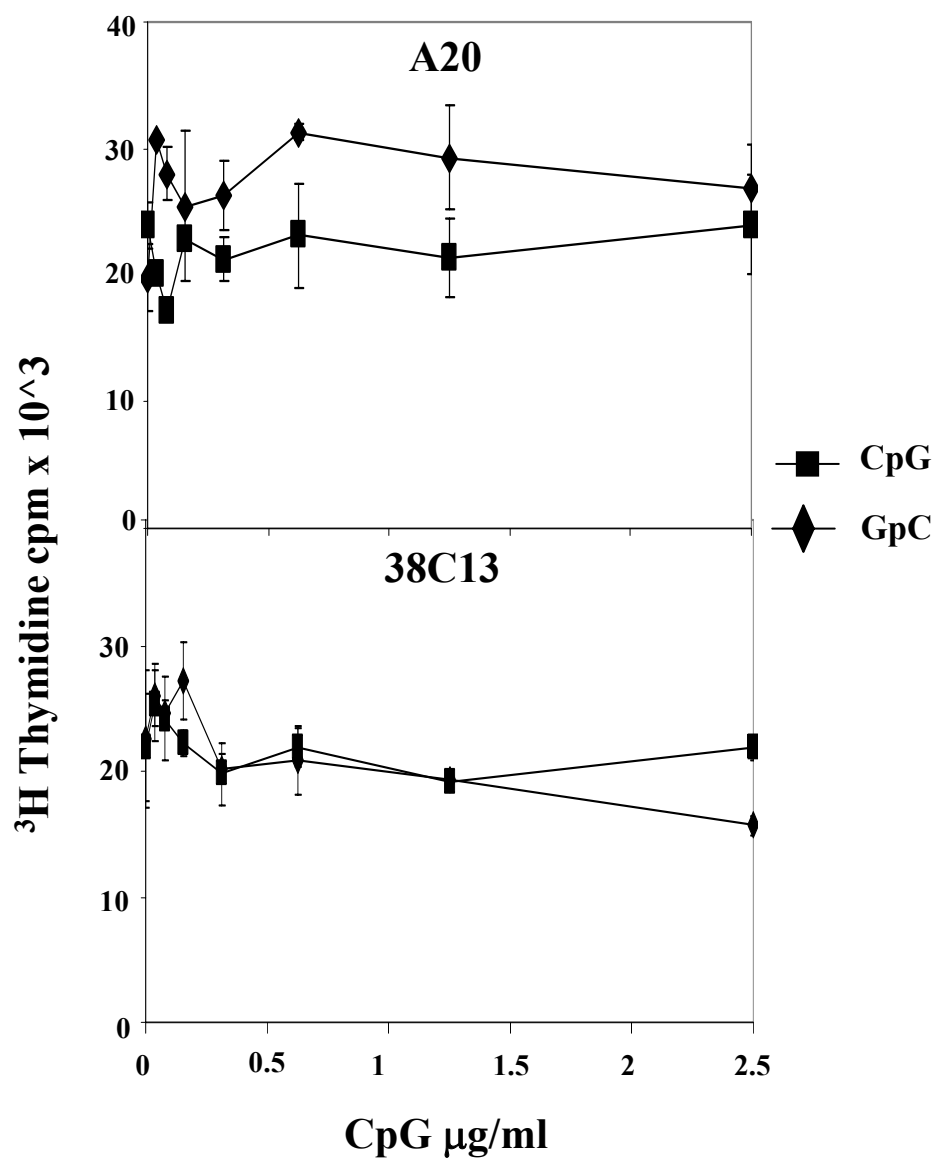
Cell proliferation and apoptosis assays were carried out as described in the Materials and Methods in Chapter II. TLR9 mRNA was amplified as described in the same section. A20 and 38C13 lymphomas did not show any significant change in proliferation upon treatment with CpG ODN compared to the untreated or GpC treated controls (Fig. A1A). Similarly, the percentages of Annexin V positive, apoptotic and unstained, live cells remained the same at all conditions studied (Fig. A1B). This indicates that CpG ODN does not induce proliferation or apoptosis in these two B cell lines. TLR9 mRNA from the B cell lymphomas was compared with that of splenic B cells. Interestingly, both cell lines expressed TLR9 mRNA, with A20 expressing relatively higher levels than the splenic B cells (Fig. A1C). It can be speculated that detecting TLR9 mRNA might not effectively translate to protein expression and account for the lack of response of these two cell lines to CpG DNA. However, I have been unsuccessful in detecting protein levels of TLR9 in splenic B cells or any cell line using mAbs currently available. Taken



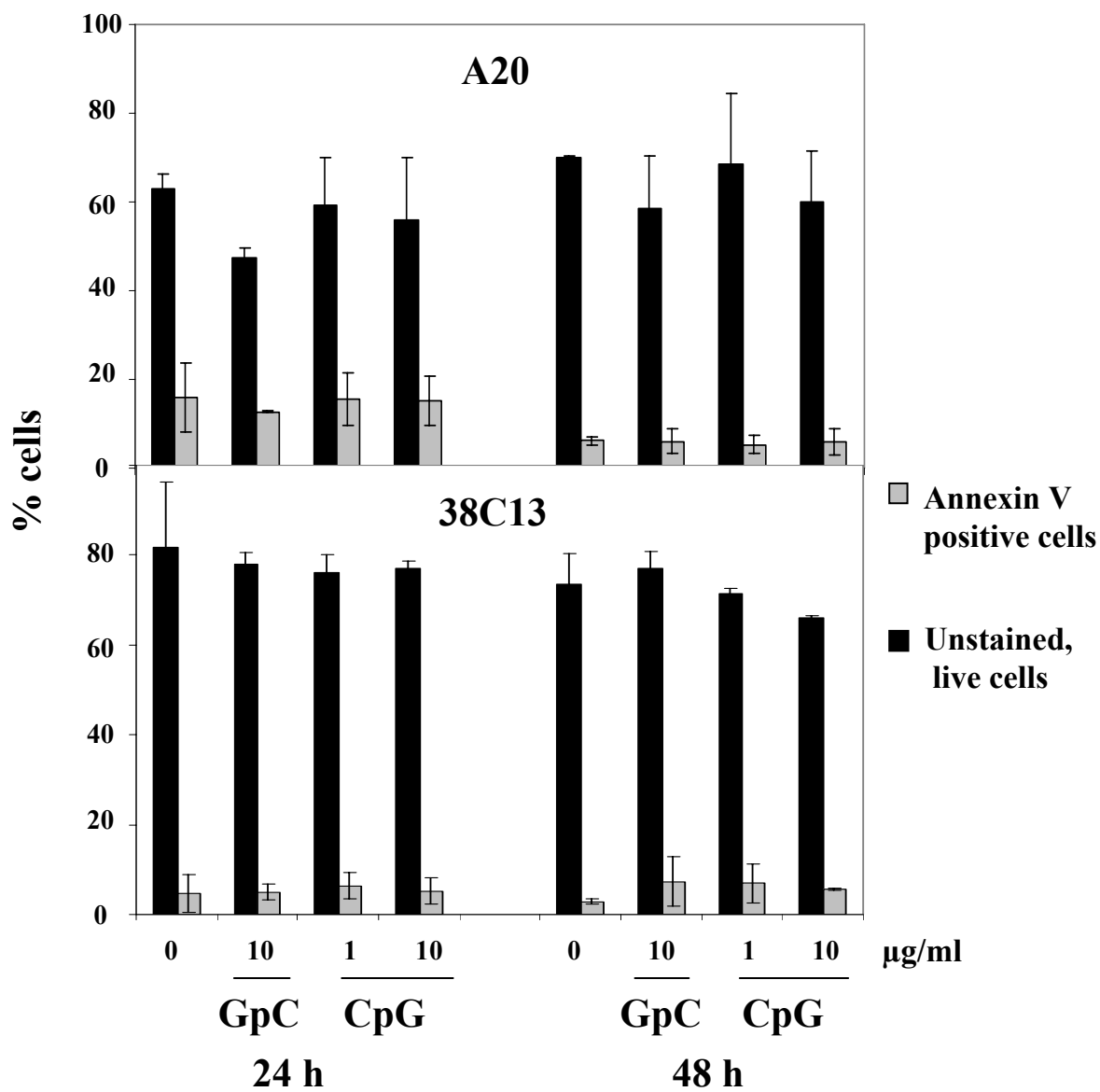
**Figure A1. Effect of CpG DNA on A20 and 38C13 B cell lymphoma lines.**

(A) A20 B cell lymphomas ( $1 \times 10^5$  cells/ml) and 38C13 B cell lymphomas ( $1 \times 10^5$  cells/ml) were treated with varying concentrations of CpG or GpC ODN for 66 hours.  $1\mu\text{Ci}$   $^3\text{H}$ - thymidine was added to each well for the last 18 hours of incubation, cells were harvested and cell associated radiation was counted using a scintillation counter. Data represents mean and S.D. of triplicate determinations. Shown are the representative results from three independent experiments. (B) A20 B cell lymphomas and 38C13 B cell lymphomas were incubated in the absence of or with varying concentrations GpC and CpG ODNs ( $1\mu\text{g/ml}$  and  $10\mu\text{g/ml}$ ) for 24 and 48 hrs. The cells were harvested, stained with Propidium Iodide (PI) and Annexin V and analyzed by flow cytometry. The percentages of unstained cells, indicating live cells (black bars) and percentages of Annexin V positive apoptotic cells (grey bars) are plotted in Figure A1B. Shown are the mean  $\pm$  S.D. from three independent experiments. (C) mRNA was isolated from the two B cell lymphoma lines and splenic lymphocytes from BALB/c mice. TLR 9 mRNA was amplified as described in Fig. 2-3A. Tubulin mRNA was also amplified using the same conditions and serves as a loading control

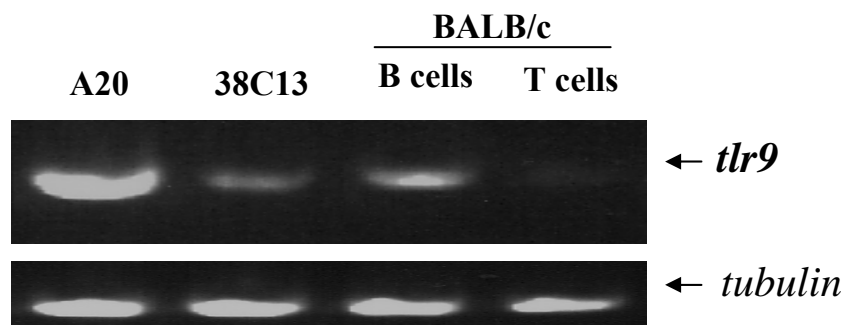
**A**



**B**



**C**



together, these data indicate that A20 and 38C13 B cell lymphomas are unresponsive to treatment with CpG ODN and this could be a function of genotypic attributes of individual cell lines.

## **Appendix B: Convergence of BCR and TLR9 signaling pathways**

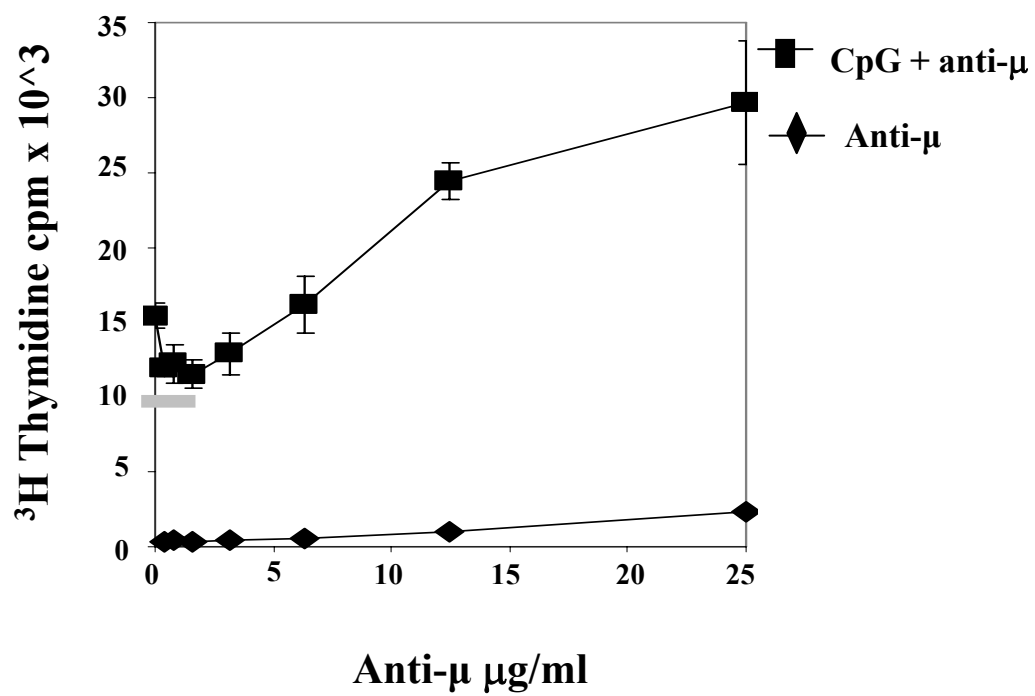
CpG ODN synergizes with BCR-induced proliferation and antibody production in mature B cells [170]. This section describes the attempt to characterize the cross-talk between BCR and TLR9 signaling pathways.

Splenic B cells were treated with varying concentrations of F(ab'2) fragments of goat anti-mouse IgM antibody alone or in combination with 0.5 µg/ml CpG ODN. Rate of proliferation was analyzed as described previously. Splenic B cells were rescued from spontaneous apoptosis and showed marginal increase in proliferation when treated with increasing concentrations of anti-Ig antibody. The proliferation of splenic B cells treated with 0.5 µg/ml CpG alone is denoted by the grey bar. When treated with 0.5 µg/ml CpG ODN and varying concentrations of anti-Ig antibodies, there was a synergistic increase in proliferation of splenic B cells (Fig. B1). This indicates a potential convergence of the BCR and TLR9 signaling pathways.

To follow the internalization of CpG ODN in CH27 lymphomas, immunofluorescence microscopy was carried out. CpG ODN was detected on the surface of the lymphomas as early as 10 min of stimulation (Fig. B2Ae and B2Be). These ODN are internalized and colocalize with LAMP-1 positive endosomal / lysosomal compartments by 30 min of stimulation (Fig. B2Bl). Cross-linking the surface BCR with anti-IgM antibodies increased the rate of CpG internalization and trafficking. At 30 min of stimulation with CpG ODN, only 7% of cells internalized the ODN, compared to 45% of cells whose surface BCR is also cross-linked. This is a significant increase in internalization of CpG

**Figure B1. Synergistic effect of CpG DNA and BCR stimulation on proliferation of primary B cells.**

Splenic B cells were treated with varying concentrations of F(ab'2) fragments of goat anti-mouse IgM antibody alone or in combination with 0.5 µg/ml CpG ODN and proliferation was analyzed as described above. The proliferation with 0.5 µg/ml CpG ODN alone is denoted by the grey bar. Data represents mean and S.D. of triplicate determinations. Shown are the representative results from three independent experiments.

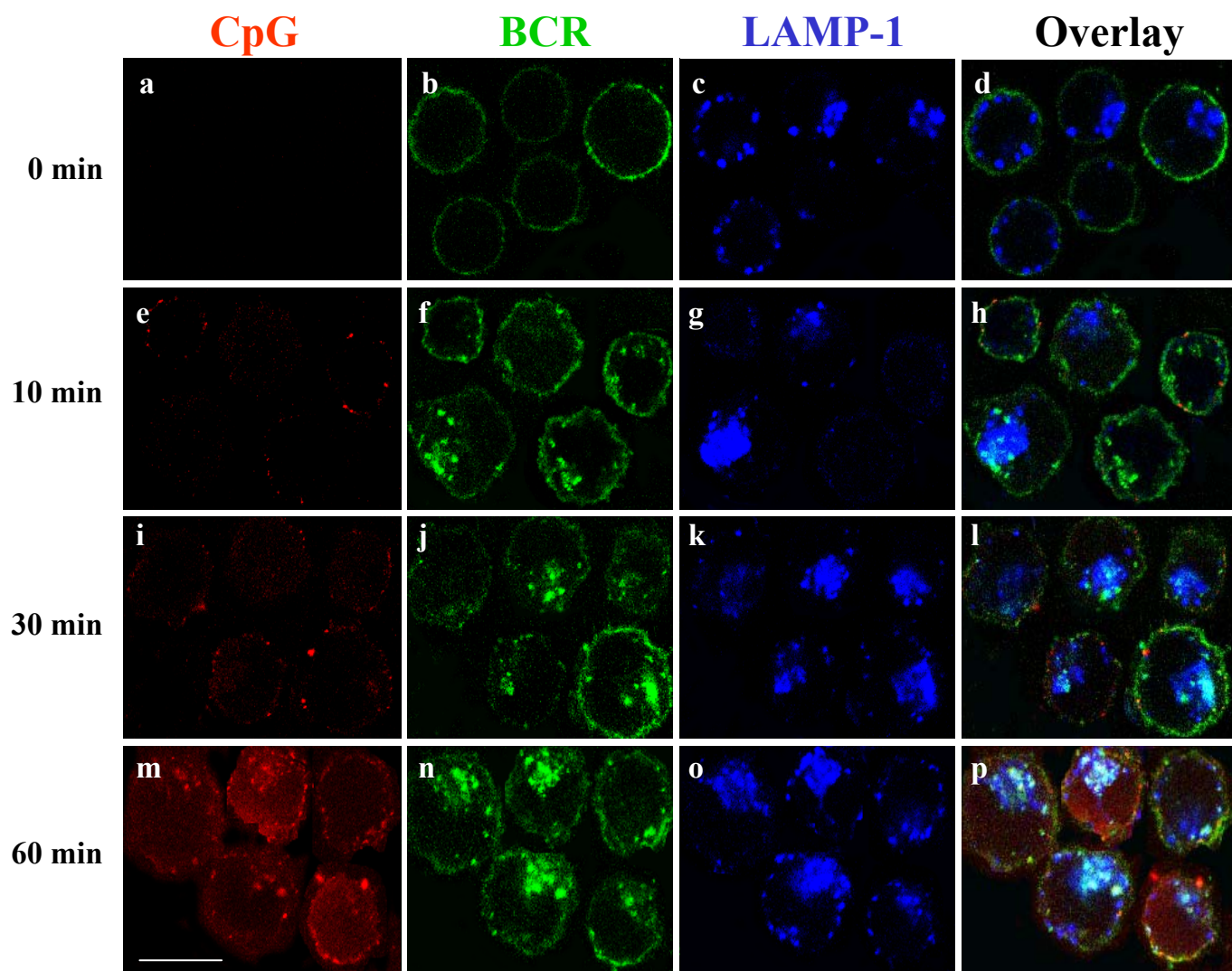


**Figure B2. Effect of cross-linking surface BCR on internalization of CpG DNA**

CH27 lymphomas were incubated with F(ab) fragments of FITC-goat anti-mouse IgM to label BCR in (A) or F(ab'2) fragments of FITC-goat anti-IgM to label and cross-link the BCR in (B). The cells were treated with 1  $\mu$ M biotinylated CpG for various times at 37°C. The cells were then fixed, permeabilized and incubated with AlexaFluor 546- streptavidin and anti-mouse LAMP-1 mAb (ID4B) followed by goat anti-mouse IgG secondary antibody. Images were acquired in the middle of the cells using a confocal fluorescence microscope. Bar, 10  $\mu$ m. (C) Shown is a representative image of cross-sectional analysis of a single cell incubated with CpG ODN for 30 min. Bar, 10  $\mu$ m. (D) Shown is a quantitative analysis of the effect of BCR cross-linking on the internalization of CpG ODN. Hundred cells were counted from five random fields of each time point of the above experiment. The percentages of cells that had internalized CpG ODN are plotted. Shown are the mean  $\pm$  S.D. from three independent experiments.

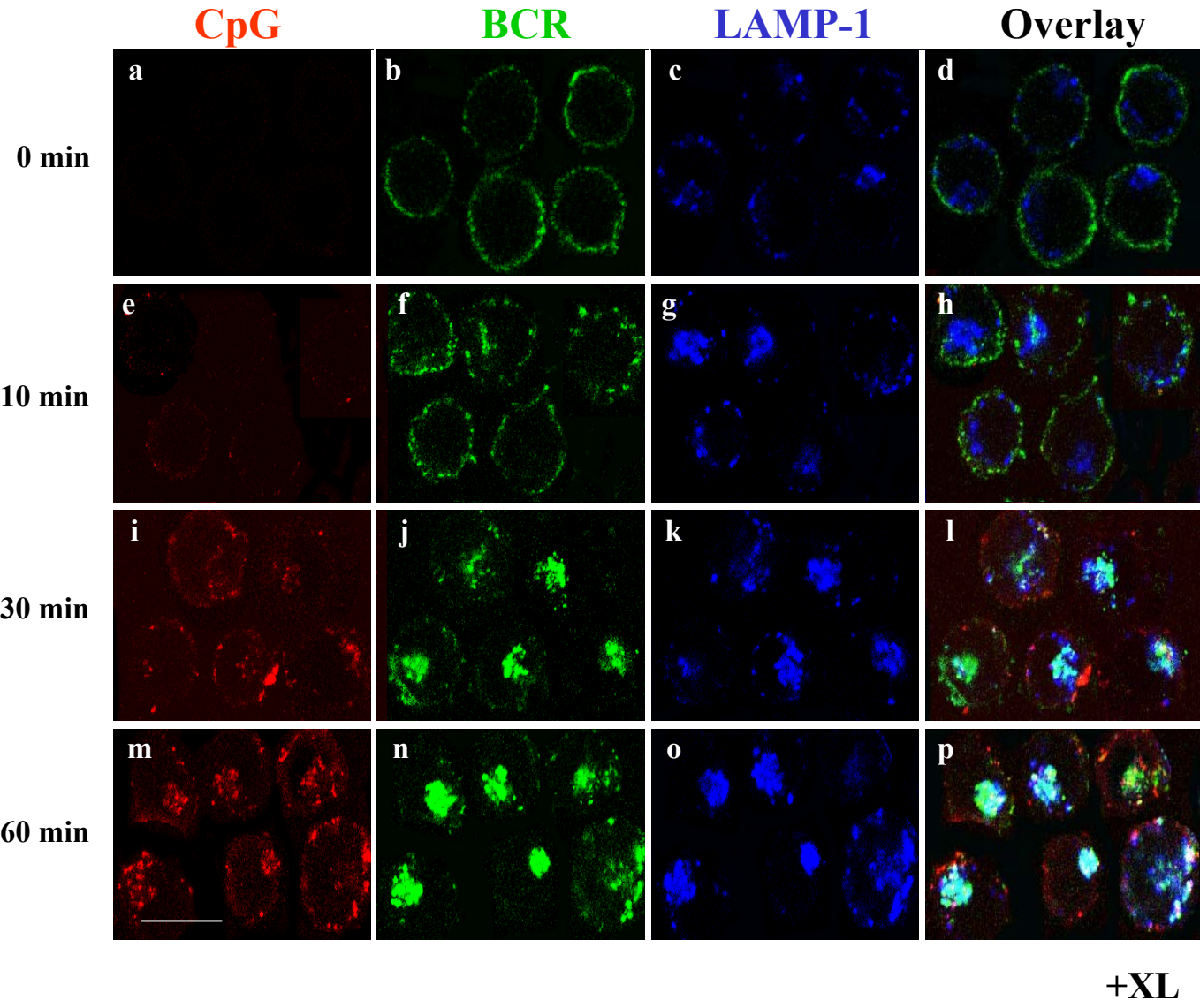


**A**

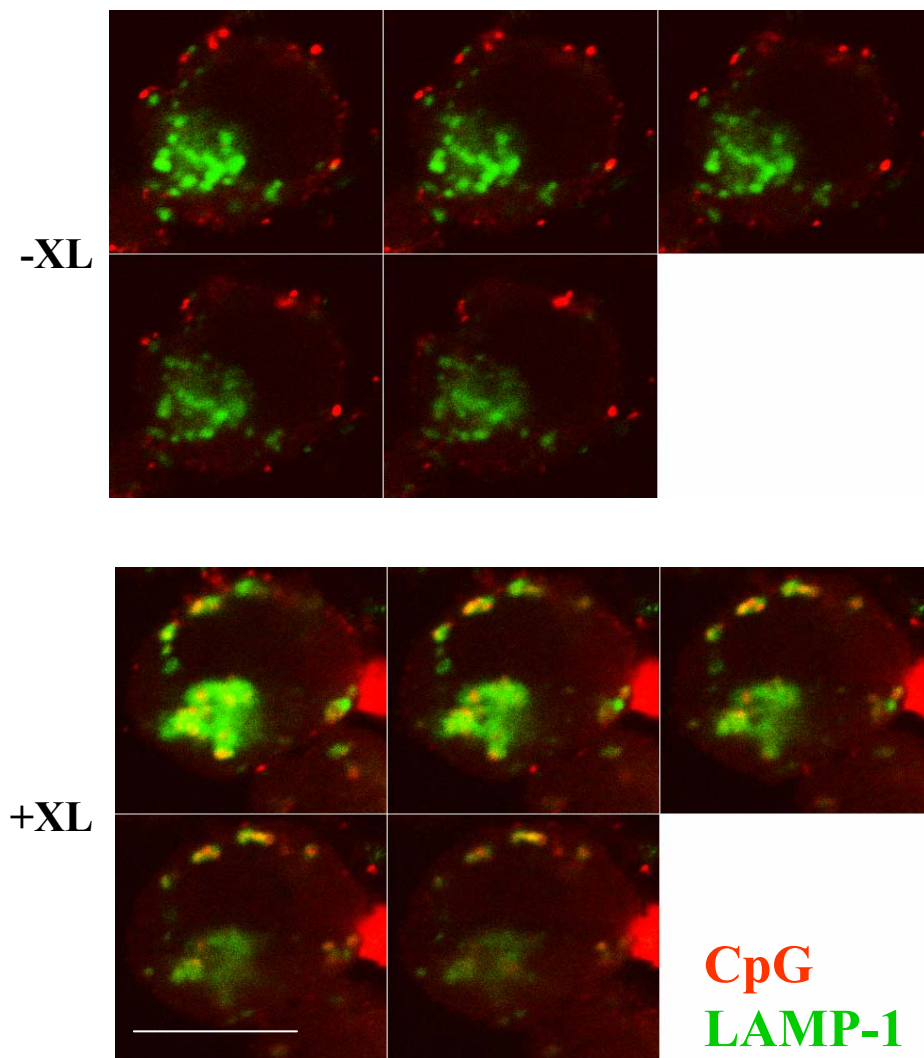


**-XL**

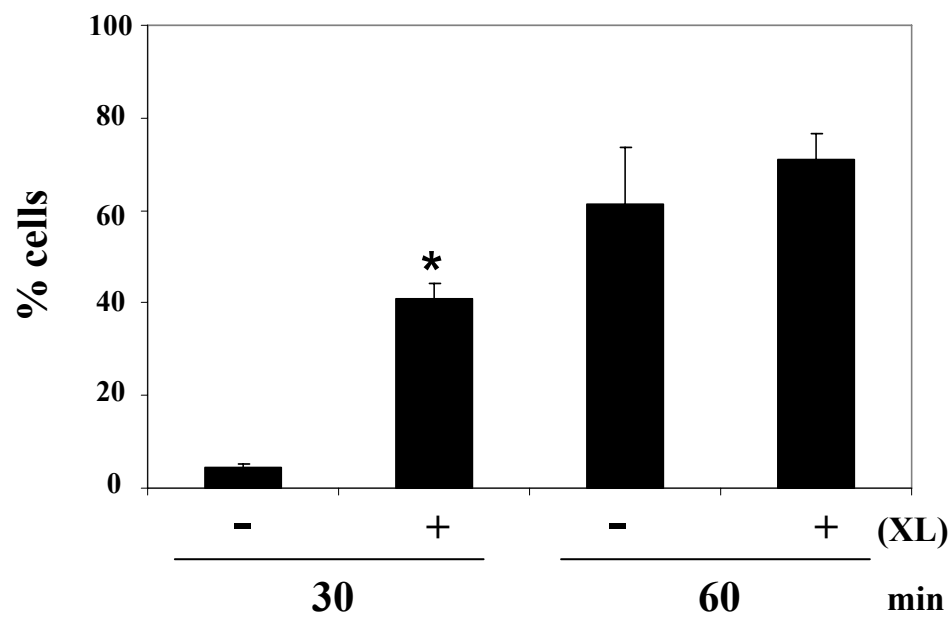
**B**



**C**



**D**



ODN by B cells BCR and TLR9 stimulation compared to the cells where only TLR9 is stimulated. At 60 min there was no significant difference in percentage of cells showing CpG internalization (Figure B2D).

Bruton's tyrosine kinase (Btk) is a member of the Tec family of kinases. Btk is critically important in development of B cells and activation and division of mature B cells [296]. Btk has been shown to interact with the TIR domains of TLRs 4, 6, 8 and 9 [297]. It is also essential for activation of NF- $\kappa$ B and TNF- $\alpha$  production by TLR4 [298]. Given the importance of Btk in B cell survival and signaling, I attempted to characterize its involvement in TLR9 signaling in mature B cells.

Splenic B cells were activated in the absence of or with 7  $\mu$ g/ml CpG for various times or anti-Ig antibody for 2 mins as positive control (P) and lysed as described in Chapter II. Tyrosine phosphorylated proteins were immunoprecipitated from the lysates using anti-phosphotyrosine mAb (4G10) antibody and protein G/Sepharose beads (Pharmacia, Piscataway, NJ). Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with goat anti-Btk and HRP-conjugated secondary antibody. Shown is a representative blot. The experiment was repeated three times with similar results.

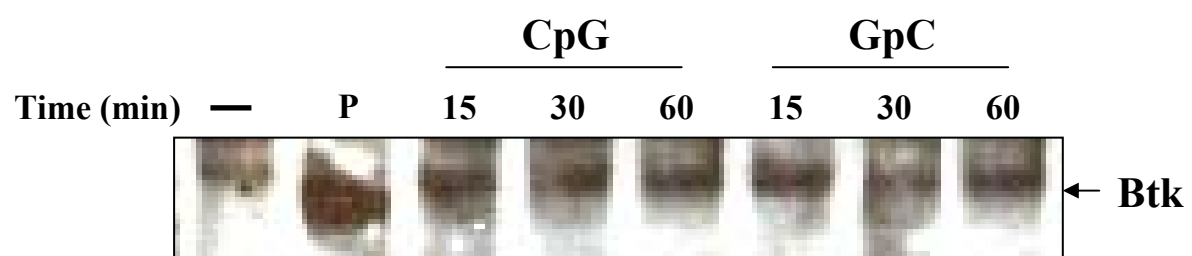
Btk is a protein tyrosine kinase which is activated by phosphorylation [29]. Hence, I analyzed the phosphorylation of Btk as an indicator of activation. A low level of phosphorylated Btk (pBtk) was detected even in the untreated samples. When the cells were treated with CpG ODN there was a slight increase in levels of pBtk, which was also

seen in samples treated with the control GpC ODN (Figure B3). Cross-linking the BCR of splenic B cells with anti-Ig antibody induced a dramatic increase in phosphorylated Btk and this serves as a positive control. Taken together, the data indicates that CpG ODN do not lead to increase in phosphorylation of Btk in mature splenic B cells over levels seen with control GpC ODN. However, this does not preclude a role for Btk in the TLR9 signaling pathway. Binding studies show that Btk can bind to the TIR domain of TLR9 [297] and hence it could potentially function as an adaptor molecule in the TLR9 pathway and not be activated by phosphorylation in B cells.

Taken together, these data indicate that the BCR and TLR9 signaling pathways are activated synergistically and signaling through the BCR enhances internalization of CpG ODN. Btk has been shown to bind to the TIR domain of TLR9, but it is not phosphorylated in response to treatment with CpG ODN in mature splenic B cells.

**Figure B3. Effect of CpG DNA on phosphorylation of Btk in B cells.**

Splenic B cells were treated in the absence of or with 7 $\mu$ g/ml of GpC and CpG ODNs for specified times. Cells were then lysed and immunoprecipitated with phosphotyrosine mAb and subjected to SDS-PAGE and Western blotting. The blots were probed with anti-Btk antibody and HRP-conjugated secondary antibody. Shown is a representative blot of three independent experiments. **P** refers to a positive control where splenic B cells were treated with goat anti-mouse IgM to cross-link surface BCR.



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